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<b>(21) International Application Number:</b> PCT/US94/02536 <b>(22) International Filing Date:</b> 9 March 1994 (09.03.94) <b>(30) Priority Data:</b> 08/034,949      22 March 1993 (22.03.93)      US <b>(71) Applicant (for all designated States except US):</b> THE UNITED STATES OF AMERICA, as represented by THE SECRETARY OF THE ARMY [US/US]; c/o Intellectual Property Counsel of the Army Office of The Judge Advocate General, DA, Suite 400, 901 North Stuart Street, Arlington, VA 22203-1837 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> REID, Robert, H. [US/US]; 10807 McComas Court, Kensington, MD 20895 (US). BOEDEKER, Edgar, C. [US/US]; 7505 Bybrook Lane, Chevy Chase, MD 20815 (US). <b>(74) Agent:</b> BELLAMY, Werten, F., W.; Intellectual Property Law Division, Office of The Judge Advocate General, Suite 400, 901 North Stuart Street, Arlington, VA 22203-1837 (US).		<b>(81) Designated States:</b> AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> VACCINES AGAINST DISEASES CAUSED BY ENTEROPATHOGENIC ORGANISMS USING ANTIGENS ENCAPSULATED WITHIN BIODEGRADABLE-BIOCOMPATIBLE MICROSPHERES  <b>(57) Abstract</b>  This invention is directed to oral parenteral and intestinal vaccines and their use against diseases caused by enteropathogenic organisms using antigens encapsulated within biodegradable-biocompatible microspheres.		

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1        VACCINES AGAINST DISEASES CAUSED BY ENTEROPATHOGENIC  
2        ORGANISMS USING ANTIGENS ENCAPSULATED WITHIN  
3        BIODEGRADABLE-BIOCOMPATIBLE MICROSPHERES

4                    I. GOVERNMENT INTEREST

5                    The invention described herein may be  
6        manufactured, licensed and used by or for governmental  
7        purposes without the payment of any royalties to us  
8        thereon.

9                    II. CROSS REFERENCE

10                   This application is a continuation-in-part of  
11        U.S. Patent Application Serial No. 07/867,301 filed  
12        April 10, 1992 which in turn is a continuation in part  
13        of U.S. Patent Application Serial No. 07/805,721 which  
14        in turn is a continuation-in-part of U.S. Patent  
15        Application Serial No. 07/690,485 filed April 27, 1991,  
16        which in turn is a continuation-in-part of U.S. Patent  
17        Application Serial No. 07/521,945 filed May 11, 1990,  
18        which in turn is a continuation-in-part of U.S. Patent  
19        Application Serial No. 07/493,597 filed March 15, 1990,  
20        which in turn is a continuation-in-part of U.S. Patent  
21        Application Serial No. 06/590,308, filed March 16,  
22        1984.

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1                                    III. FIELD OF THE INVENTION

2                                    This invention relates to parenteral and  
3                                    oral-intestinal vaccines against diseases caused by  
4                                    enteropathogenic organisms using antigens encapsulated  
5                                    within biodegradable-biocompatible microspheres  
6                                    (matrix).

7                                    PHASE I

8                                    IV. BACKGROUND OF THE INVENTION

9                                    Most infectious agents have their first  
10                                   contact with the host at a mucosal surface; therefore,  
11                                   mucosal protective immune mechanisms are of primary  
12                                   importance in preventing these agents from colonizing  
13                                   or penetrating the mucosal surface. Numerous studies  
14                                   have demonstrated that a protective mucosal immune  
15                                   response can best be initiated by introduction of the  
16                                   antigen at the mucosal surface, and parenteral  
17                                   immunization is not an effective method to induce  
18                                   mucosal immunity. Antigen taken up by the  
19                                   gut-associated lymphoid tissue (GALT), primarily by the  
20                                   Peyer's patches in mice, stimulates T helper cell ( $T_H$ )  
21                                   to assist in IgA B cell responses or stimulates T  
22                                   suppressor cells ( $T_s$ ) to mediate the unresponsiveness  
23                                   of oral tolerance. Particulate antigen appears to  
24                                   shift the response towards the ( $T_H$ ) whereas soluble  
25                                   antigens favor a response by the ( $T_s$ ). Although



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1 studies have demonstrated that oral immunization does  
2 induce an intestinal mucosal immune response, large  
3 doses of antigen are usually required to achieve  
4 sufficient local concentrations in the Peyer's  
5 patches. Unprotected protein antigens may be degraded  
6 or may complex with secretory IgA in the intestinal  
7 lumen.

8 One possible approach to overcoming these  
9 problems is to homogeneously disperse the antigen of  
10 interest within the polymeric matrix of appropriately  
11 sized biodegradable, biocompatible microspheres that  
12 are specifically taken up by GALT. Eldridge et. al.  
13 have used a murine model to show that  
14 orally-administered 1-10 micrometer microspheres  
15 consisting of polymerized lactide and glycolide, (the  
16 same materials used in resorbable sutures), were  
17 readily taken up into Peyer's patches, and the 1-5  
18 micrometer size were rapidly phagocytized by  
19 macrophages. Microspheres that were 5-10 micrometers  
20 (microns) remained in the Peyer's patch for up to 35  
21 days, whereas those less than 5 micrometer disseminated  
22 to the mesenteric lymph node (MLN) and spleen within  
23 migrating MAC-1<sup>+</sup> cells. Moreover, the levels of  
24 specific serum and secretory antibody to staphylococcal  
25 enterotoxin B toxoid and inactivated influenza A virus  
26 were enhanced and remained elevated longer in animals

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1        which were immunized orally with microencapsulated  
2        antigen as compared to animals which recieved equal  
3        doses of non-encapsulated antigen. These data indicate  
4        that microencapsulation of an antigen given orally may  
5        enhance the mucosal immune response against enteric  
6        pathogens. AF/R1 pili mediate the species-specific  
7        binding of E. coli RDEC-1 with mucosal glycoproteins  
8        in the small intestine of rabbits and are therefore an  
9        important virulence factor. Although AF/R1 pili are  
10       not essential for E. coli RDEC-1 to produce  
11       enteropathogenic disease, expression of AF/R1 promotes  
12       a more severe disease. Anti-AF/R1 antibodies have  
13       been shown to inhibit the attachment of RDEC-1 to the  
14       intestinal mucosa and prevent RDEC-1 disease in  
15       rabbits. The amino acid sequence of the AF/R1 pilin  
16       subunit has recently been determined, but specific  
17       antigenic determinants within AF/R1 have not been  
18       identified.

19                Recent advances in the understanding of B  
20       cell and T cell epitopes have improved the ability to  
21       select probably linear epitopes from the amino acid  
22       sequence using theoretical criteria. B cell epitopes  
23       are often composed of a string of hydrophilic amino  
24       acids with a high flexibility index and a high  
25       probability of turns within the peptide structure.  
26       Prediction of T cell epitopes are based on the Rothbard

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1 method which identifies common sequence patterns that  
2 are common to known T cell epitopes or the method of  
3 Berzofsky and others which uses a correlation between  
4 algorithms predicting amphipathic helices and T cell  
5 epitopes.

6 In the current study we have used these  
7 theoretical criteria to predict probable T or B cell  
8 epitopes from the amino acid sequence of AF/R1. Four  
9 different 16 amino acid peptides that include the  
10 predicted epitopes have been synthesized: AF/R1 40-55  
11 as a B cell epitope, 79-94 as a T cell epitope, 108-123  
12 as a T and B cell epitope, and AF/R1 40-47/79-86 as a  
13 hybrid of the first eight amino acids from the  
14 predicted B cell epitope and the T cell epitope. We  
15 have used these peptides as well as the native protein  
16 to stimulate the in vitro proliferation of lymphocytes  
17 taken from the Peyer's patch, MLN, and spleen of  
18 rabbits which have recieved intraduodenal priming with  
19 microencapsulated or non-encapsulated AF/R1. Our  
20 results demonstrate the microencapsulation of AF/R1  
21 potentiates the cellular immune response at the level  
22 of the Peyer's patch, thus enhancing in vitro  
23 lymphocyte proliferation to both the native protein and  
24 its linear peptide antigens. CFA/I pili, rigid  
25 thread-like structures which are composed of repeating  
26 pilin subunits of 147 amino acid found on serogroups

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1        015, 025, 078, and 0128 of enterotoxigenic E. coli  
2        (ETEC) [1-4, 18]. CFA/I promotes mannose resistant  
3        attachment to human brush borders [5]; therefore, a  
4        vaccine that established immunity against this protein  
5        may prevent the attachment to host tissues and  
6        subsequent disease. In addition, because the CFA/I  
7        subunit shares N-terminal amino acid sequence homology  
8        with CS1, CFA/II (CS2) and CFA/IV (CS4) [4], a subunit  
9        vaccine which contained epitopes from this area of the  
10       molecule may protect against infection with various  
11       ETEC.

12                Until recently, experiments to identify these  
13        epitopes were time consuming and costly; however,  
14        technology is now available which allows one to  
15        simultaneously identify all the T cell and B cell  
16        epitopes in the protein of interest. Multiple Peptide  
17        synthesis (Pepscan) is a technique for the simultaneous  
18        synthesis of hundreds of peptides on polyethylene rods  
19        [6]. We have used this method to synthesize all the  
20        140 possible overlapping actapeptides of the CFA/I  
21        protein. The peptides, still on the rods, can be used  
22        directly in ELISA assays to map B cell epitopes [6,  
23        12-14]. We have also synthesized all the 138 possible  
24        overlapping decapeptides of the CFA/I protein. For  
25        analysis of T cell epitopes, these peptides can be  
26        cleaved from the rods and used in proliferation assays

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[15]. Thus this technology allows efficient mapping and localization of both B cell and T cell epitopes to a resolution of a single amino acid [16]. These studies were designed to identify antigenic epitopes of ETEC which may be employed in the construction of an effective subunit vaccine.

CFA/I pili consist of repeating pilin protein subunits found on several serogroups of enterotoxigenic E. coli (ETEC) which promote attachment to human intestinal mucosa. We wished to identify areas within the CFA/I molecule that contain immunodominant T cell epitopes that are capable of stimulating the cell-mediated portion of the immune response in primates as well as immunodominant B cell epitopes. To do this, we (a) resolved the discrepancy in the literature on the complete amino acid sequence of CFA/I, (b) immunized three Rhesus monkeys with multiple i.m. injections of purified CFA/I subunit in Freund's adjuvant, (c) synthesized 138 overlapping decapeptides which represented the entire CFA/I protein using the Pepscan technique (Cambridge Research Biochemicals), (d) tested each of the peptides for their ability to stimulate the spleen cells from the immunized monkeys in a proliferative assay (e) synthesized 140 overlapping octapeptides which represented the entire CFA/I protein, and (f) tested

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1 serum from each monkey for its ability to recognize the  
2 octapeptides in a modified ELISA assay. A total of 39  
3 different CFA/I decapeptides supported a significant  
4 proliferative response with the majority of the  
5 responses occurring within distinct regions of the  
6 protein (peptides beginning with residues 8-40, 70-80,  
7 and 127-137). Nineteen of the responsive peptides  
8 contained a serine residue at positions 2, 3, or 4 in  
9 the peptide, and a nine contained a serine specifically  
10 at position 3. Most were predicted to be configured as  
11 an alpha helix and have a high amphipathic index.  
12 Eight B cell epitopes were identified at positions  
13 3-11, 11-21, 22-29, 32-40, 38-45, 66-74, 93-101, and  
14 124-136. The epitope at position 11-21 was strongly  
15 recognized by all three individual monkeys, while the  
16 epitopes at 93-101, 124-136, 66-74, and 22-29 were  
17 recognized by two of the three monkeys.

#### 18 V. SUMMARY OF THE INVENTION

19 This invention relates to a novel  
20 pharmaceutical composition, a microcapsule/sphere  
21 formulation, which comprises an antigen encapsulated  
22 within a biodegradable polymeric matrix such as poly  
23 (DL-lactide-co-glycolide) (DL-PLG), wherein the  
24 relative ratio between the lactide and glycolide  
25 component of the DL-PLG is within the range of 40:60 to  
26 0:100, and its use, as a vaccine, in the effective

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1 pretreatment of animals (including humans) to prevent  
2 intestinal infections caused by a virus or bacteria.  
3 In the practice of this invention, applicants found  
4 that the AF/R1 adherence factor is a plasmid encoded  
5 pilus composed of repeating pilin protein subunits  
6 that allows E. coli RDEC-1 to attach to rabbit  
7 intestinal brush borders. To identify an approach that  
8 enhances the immunogenicity of antigens that contact  
9 the intestinal mucosa, applicants investigated the  
10 effect of homogeneously dispersing AF/R1 pili within  
11 biodegradable microspheres that included a size range  
12 selected for Peyer's Patch localization. New Zealand  
13 White rabbits were primed twice with 50 micrograms of  
14 either microencapsulated or nonencapsulated AF/R1 by  
15 endoscopic intraduodenal inoculation. Lymphoid tissues  
16 were removed and cellular proliferative responses to  
17 AF/R1 and synthetic AF/R1 peptides were measured in  
18 vitro. The synthetic peptides represented possible T  
19 and/or B cell epitopes which were selected from the  
20 AF/R1 subunit sequence using theoretical criteria. In  
21 rabbits which had received nonencapsulated AF/R1,  
22 Peyer's Patch cells demonstrated slight but significant  
23 proliferation in vitro in response to AF/R1 pili but  
24 not the AF/R1 synthetic peptides. In rabbits which had  
25 received microencapsulated AF/R1, Peyer's Patch cells  
26 demonstrated a markedly enhanced response to AF/R1 and

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1 the synthetic peptides. Cells from the spleen and  
2 mesenteric lymph nodes responded similarly to AF/R1  
3 pili in both groups of animals, while there was a  
4 greater response to the synthetic peptide AF/R1 40-55  
5 in rabbits that had received microencapsulated AF/R1.  
6 These data demonstrate that microencapsulation of AF/R1  
7 potentiates the mucosal cellular immune response to  
8 both the native protein and its linear peptide  
9 antigens.

#### 10 VI. BRIEF DESCRIPTION OF THE DRAWINGS

11 Figure 1 shows the size distribution of  
12 microspheres wherein the particle size distribution (%)  
13 is (a) By number 1-5 (91) and 6-10 (9) and (b) By  
14 weight 1-5 (28) and 6-10 (72).

15 Figure 2 shows a scanning electron micrograph  
16 of microspheres.

17 Figures 3(a) and (b) show the In vitro  
18 immunization of spleen cells and demonstrates that  
19 AF/RI pilus protein remains immunogenic to rabbit  
20 spleen cells immunized in vitro after  
21 microencapsulation. AF/R1 pilus protein has been found  
22 to be immunogenic for rabbit spleen mononuclear cells  
23 in vitro producing a primary IgM antibody response  
24 specific to AF/RI. Immunization with antigen  
25 encapsulated in biodegradable, biocompatible  
26 microspheres consisting of lactide/glycolide copolymers



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1 has been shown to endow substantially enhanced immunity  
2 over immunization with the free antigen. To determine  
3 if microencapsulated AF/RI maintains the immunogenicity  
4 of the free pilus protein, a primary in vitro  
5 immunization assay was conducted. Rabbit spleen  
6 mononuclear cells at a concentration of  $3 \times 10^5$   
7 cells/well. Triplicate wells of cells were immunized  
8 with free AF/RI in a dose range from 15 to 150 ng/ml or  
9 with equivalent doses of AF/RI contained in  
10 microspheres. Supernatants were harvested on days 7,  
11 9, 12, and 14 of culture and were assayed for free  
12 AF/RI pilus protein specific IgM antibody by the ELISA.  
13 Supernatant control values were subtracted from those  
14 of the immunized cells. Cells immunized with free  
15 pilus protein showed a significant positive IgM  
16 response on all four days of harvest, with the antibody  
17 response increasing on day 9, decreasing on day 12, and  
18 increasing again on day 14. Cells immunized with  
19 microencapsulated pilus protein showed a comparable  
20 positive IgM antibody response as cells immunized with  
21 free pilus protein. In conclusion, AF/RI maintains  
22 immunogenicity to rabbit spleen cells immunized in  
23 vitro after microencapsulation.

24 Figures 4(a) and (b) show in vitro  
25 immunization of Peyer's patch cells. Here the AF/RI  
26 pilus protein remains immunogenic to rabbit Peyer's

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1 patch cells immunized in vitro after  
2 microencapsulation. AF/RI pilus protein has been found  
3 to be immunogenic for rabbit Peyer's patch mononuclear  
4 cells in vitro producing a primary IgM antibody  
5 response specific to AF/RI. Immunization with antigen  
6 encapsulated in biodegradable, biocompatible  
7 microspheres consisting of lactide/glycolide copolymers  
8 has been shown to endow substantially enhanced immunity  
9 over immunization with the free antigen. To determine  
10 if microencapsulated AF/RI maintains the immunogenicity  
11 of the free pilus protein, a primary in vitro  
12 immunization assay was conducted. Rabbit Peyer's patch  
13 mononuclear cells at a concentration of  $3 \times 10^6$  cells/ml  
14 were cultured in 96-well, round bottom microculture  
15 plates at a final concentration of  $6 \times 10^5$  cells/well.  
16 Triplicate wells of cells were immunized with free  
17 AF/RI in a dose range from 15 to 150 ng/ml or with  
18 equivalent dose of AF/RI contained in microspheres.  
19 Supernatants were harvested on days 7, 9, 12, and 14 of  
20 culture and were assayed for free AF/RI pilus protein  
21 specific IgM antibody by the ELISA. Supernatant  
22 control values were subtracted from those of the  
23 immunized cells. Cells immunized with free pilus  
24 protein showed a significant positive IgM response on  
25 all four days of harvest, with the highest antibody  
26 response on day 12 with the highest antigen dose.

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1 Cells immunized with encapsulated pilus protein showed  
2 a positive response on day 12 with all three antigen  
3 doses. In conclusion, AF/RI pilus protein maintains  
4 immunogenicity to rabbit Peyer's patch cells immunized  
5 in vitro after microencapsulation.

6 Figure 5 shows proliferative responses to  
7 AF/RI by rabbit Peyer's patch cells. Naive rabbits  
8 were primed twice with 50 micrograms of either  
9 non-encapsulated (rabbits 132 and 133) or  
10 microencapsulated (rabbits 134 and 135) AF/RI pili by  
11 endoscopic intraduodenal inoculation seven days apart.  
12 Seven days following the second priming, Peyer's patch  
13 cells were cultured with AF/RI in 96-well plates for  
14 four days followed by a terminal six hour pulse with  
15 [<sup>3</sup>H]thymidine. Data shown is the SI calculated from  
16 the mean cpm of quadruplicate cultures. Responses were  
17 significant for all rabbits: 132 (p=0.013), 133  
18 (p=.0006), 134 (p=0.0016), and 135 (p=0.0026).  
19 Responses were significantly different between the two  
20 groups. Comparison of the best responder in the  
21 nonencapsulated antigen group (rabbit 133) with the  
22 lowest responder in the microencapsulated antigen group  
23 (rabbit 134) demonstrated an enhanced response when the  
24 immunizing antigen was microencapsulated (p=0.0034).

25 Additionally, Figure 5 relates to the in  
26 vitro lymphocyte proliferation after sensitization of

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1 rabbit lymphoid tissues with encapsulated or  
2 non-encapsulated AF/RI pilus adhesion of E. coli strain  
3 RDEC-1. The AF/RI adherence factor is a plasmid  
4 encoded pilus protein that allows RDEC-1 to attach to  
5 rabbit intestinal brush borders. We investigated the  
6 immunopotentiating effect of encapsulating purified  
7 AF/RI into biodegradable non-reactive microspheres  
8 composed of polymerized lactide and glycolide,  
9 materials used in resorbable sutures. The microspheres  
10 had a size range of 5-10 microns, a size selected for  
11 Peyer's Patch localization, and contained 0.62% protein  
12 by weight. NZW rabbits were immunized twice with 50  
13 micrograms of either encapsulated or non-encapsulated  
14 AF/RI by intraduodenal later of non-encapsulated AF/RI  
15 by intraduodenal inoculation seven days apart.  
16 Lymphocyte proliferation in response to purified AR/RI  
17 was conducted in vitro at seven days and showed that  
18 encapsulating the antigen into microspheres enhanced  
19 the cellular immune response in the Peyer's Patch;  
20 however, no significant increase was observed in spleen  
21 or mesenteric lymph node. These data suggest that  
22 encapsulation of AF/RI may potentiate the mucosal  
23 cellular immune response.

24 Figures 6 a-d show proliferative responses to  
25 AF/RI synthetic peptides by rabbit Peyer's patch  
26 cells. Naive rabbits were primed twice with 50

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1 micrograms of either non-encapsulated (rabbits 132 and  
2 133) or microencapsulated (rabbits 134 and 135) AF/RI  
3 pili by endoscopic intraduodenal inoculation seven days  
4 apart. Seven days following the second priming,  
5 Peyer's patch cells from each rabbit were cultured with  
6 AF/RI 40-55 (Fig. 6a), AF/RI 79-94 (Fig. 6b), AF/RI  
7 108-123 (Fig 6c), or AF/RI 40-47/79-86 (Fig. 6d) in  
8 96-well plates for four days followed by a terminal six  
9 hour pulse with [<sup>3</sup>H]thymidine. Data shown is the SI  
10 calculated from the mean cpm of quadruplicate cultures.  
11 The responses of rabbits 132 and 133 were not  
12 significant to any of the peptides tested. Rabbit 134  
13 had a significant response to (a) AF/RI 40-55  
14 (p=0.0001), (b) AF/RI 79-94 (p=0.0280), and (d) AF/RI  
15 40-57/79-86 (p=0.025), but not to (c) AF/RI 108-123.  
16 Rabbit 135 had a significant response to (a) AF/RI  
17 40-55 (p=0.034), (b) AF/RI 79-94 (p=0.040), and (c)  
18 AF/RI 108-123 (p<0.0001), but not to (d) AF/RI  
19 40-47/79-86. This demonstrates enhanced proliferative  
20 response to peptide antigens following mucosal priming  
21 with microencapsulated pili. AF/RI pili promotes  
22 RDEC-1 attachment to rabbit intestinal brush borders.  
23 Three 16 amino acid peptides were selected by  
24 theoretical criteria from the AF/RI sequence as  
25 probable T or B cell epitopes and were synthesized:  
26 AF/RI 40-55 as a B cell epitope, 79-94 as a T cell

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1 epitope, and 108-123 as a T and B cell epitope. We  
2 used these peptides to investigate a possible  
3 immunopotentiating effect of encapsulating purified  
4 Af/RI pili into biodegradable, biocompatible  
5 microspheres composed of polymerized lactide and  
6 glycolide at a size range that promotes localization in  
7 the Peyer's Patch (5-10 micrometers). NZW rabbits  
8 were primed twice with 50 micrograms AF/RI by  
9 endoscopic intraduodenal inoculation and their Peyer's  
10 Patch cells were cultured in vitro with the AF/RI  
11 peptides. In two rabbits which had received  
12 encapsulated AF/RI, lymphocyte proliferation was  
13 observed to AF/RI 40-55 and 79-94 in both rabbits and  
14 to 108-123 in one of two rabbits. No responses to any  
15 of the peptides were observed in rabbits which received  
16 non-encapsulated AF/RI. These data suggest that  
17 encapsulation of AF/RI may enhance the cellular  
18 response to peptide antigens.

19 Figures 7a-d show B-cell responses of Peyer's  
20 patch cells to AF/R1 and peptides.

21 Figures 8a-d show B-cell responses of Peyer's  
22 Patch cells to AF/R1 and peptides.

23 Figures 9a-d show B-cell responses of spleen  
24 cells to AF/R1 and Peptides.

25 Figures 10a-d show B cell responses of spleen  
26 cells to AF/R1 and peptides.

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1                   Figures 7 through 10, illustrate enhanced  
2                   lymphocyte antibody response by mucosal immunization of  
3                   rabbits with microencapsulated AF/R1 pilus protein.  
4                   The AF/R1 pilus protein has been found to be  
5                   immunogenic for rabbit spleen and Peyer's patch cells  
6                   in vitro producing a primary IgM antibody response.  
7                   The purpose of this study was to determine if AR/R1  
8                   pilus protein immune response is enhanced by  
9                   microencapsulation. The AF/R1 was incorporated into  
10                  biodegradable, biocompatible microspheres composed of  
11                  lactide-glycolide copolymers, had a size range of 5-10  
12                  micrometer and containing 0.62% pilus protein by  
13                  weight. Initially, NZW rabbits were immunized twice  
14                  with 50 micrograms of either encapsulated or  
15                  non-encapsulated AF/R1 via intraduodenal route seven  
16                  days apart. For in vitro challenge,  $6 \times 10^5$  rabbit  
17                  lymphocytes, were set in microculture at final volume  
18                  of 0.2 ml. Cells were challenged with AR/R1 or three  
19                  different synthetic 16 amino acid peptides  
20                  representing, either predicted T, B or T and B cell  
21                  epitopes in a dose range of 15 to 150 ng/ml for splenic  
22                  cells or 0.05 to 5.0 micrograms/ml for Peyer's patch  
23                  mononuclear cells (in triplicate). Supernatants were  
24                  collected on culture days 3, 5, 7, and 9 assayed by  
25                  ELISA for anti-AF/R1 antibody response as compared to  
26                  cell supernatant control. Significant antibody

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1 responses were seen only from spleen and Peyer's patch  
2 cells from rabbits immunized with microencapsulated  
3 AF/R1. The antibody response tended to peak between  
4 days 5 and 9 was mainly an IgM response. The results  
5 for the predicted epitopes were similar to those  
6 obtained with purified AF/R1. In conclusion,  
7 intestinal immunization with AF/R1 pilus protein  
8 contained within microspheres greatly enhances both the  
9 spleen and Peyer's patch B-cell responses to predicted  
10 T & B-cell epitopes.

11 Figure 11 shows proliferative responses to  
12 AF/R1 40-55 by rabbit MLN cells. Naive rabbits were  
13 primed twice with 50 micrograms of either  
14 nonencapsulated (rabbits 132 and 133) or  
15 microencapsulated (rabbits 134 and 135) AF/R1 pili by  
16 endoscopic intraduodenal inoculation seven days apart.  
17 Seven days following the second priming, MLN cells were  
18 cultured with AF/R1 40-55 for four days in 24-well  
19 plates. Cultures were transferred into 96-well plates  
20 for a terminal [<sup>3</sup>H]thymidine pulse. Data shown is the  
21 SI calculated from the mean cpm of quadruplicate  
22 cultures. Responses of rabbits 132 and 133 were not  
23 statistically significant. Responses were significant  
24 for rabbits 134 ( $p=0.0051$ ) and 135 ( $p=0.0055$ ).

25 Figure 12 shows proliferative responses to  
26 AF/R1 40-55 by rabbit spleen cells. Naive rabbits were



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1        primed twice with 50 micrograms of either  
2        nonencapsulated (rabbits 132 and 133) or  
3        microencapsulated (rabbits 134 and 135) AF/R1 pili by  
4        endoscopic intraduodenal inoculation seven days apart.  
5        Seven days following the second priming, spleen cells  
6        were cultured with AF/R1 40-55 for four days in 24-well  
7        plates. Cultures were transferred into 96 well plates  
8        for a terminal [<sup>3</sup>H]thymidine pulse. Data shown is the  
9        SI calculated from the mean cpm of quadruplicate  
10       cultures. Responses of rabbits 132 and 133 were not  
11       statistically significant. Responses were significant  
12       for rabbits 134 (p=0.0.0005) and 135 (p=0.0066).

13                Figure 16. A. SDS-PAGE of intact CFA/I (lane  
14        1), trypsin treated CFA/I (lane 2), and *S. aureus* V8  
15        protease treated CFA/I. Molecular masses of individual  
16        bands were estimated from molecular weight standards  
17        (on left). Multiple lanes of both trypsin and V8  
18        treated CFA/I were transferred to PVDF membranes where  
19        bands corresponding to the approximate molecular masses  
20        of 3500 (trypsin digest, see arrow lane 2) and 6000 (V8  
21        digest, see arrow lane 3) were excised and subjected to  
22        Edman degradation. B. Resulting sequence of protein  
23        fragments from each lane of A (position of sequenced  
24        portion of fragment in the intact protein. Underlined,  
25        italicized residues are amino acids under dispute in  
26        literature.

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1                   Figure 17. ELISA assay results testing  
2           hyperimmune sera of monkeys (A)2Z2 (monkey 3), (B)  
3           184(D) (monkey 1) and (C) 34 (monkey 2) to CFA/I  
4           primary structure immobilized on polyethylene pins.  
5           Monkey sera diluted 1:1000. Peptide number refers  
6           first amino acid in sequence of octapeptide on pin from  
7           CFA/I primary structure OD 405 refers to optical  
8           density wavelength at which ELISA plates were read (405  
9           nm).

10                   Figure 18. Complete sequence of CFA/I (147  
11           amino acids) with B cell recognition site (boxed areas)  
12           as defined by each individual monkey response (2Z2,  
13           184D, and 34). Derived from data in Figure 17.

14                   Figures 19-21. Lymphocyte proliferation to  
15           synthetic decapeptides of CFA/I. Each monkey was  
16           immunized with three i.m. injections of CFA/I subunits  
17           in adjuvant, and its spleen cells were cultured with  
18           synthetic decapeptides which had been constructed using  
19           the Pepsan technique. The decapeptides represented  
20           the entire CFA/I protein. Concentrations of synthetic  
21           peptide used included 6.0, 0.6, and 0.06 micrograms/ml.  
22           Values shown represent the maximum proliferative  
23           response produced by any of the three concentrations of  
24           antigen used  $\pm$  the standard deviation. The cpm of the  
25           control peptide for each of the three monkeys was 1,518  
26            $\pm$  50, 931  $\pm$  28, and 1,553  $\pm$  33 respectively. The cpm

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1 of the media control for each of the three monkeys was  
2  $1,319 \pm 60$ ,  $325 \pm 13$ , and  $1,951 \pm 245$  respectively.

3 Figures 22-24. Lymphocyte proliferation to  
4 6.0, 0.6, and 0.06 micrograms/ml synthetic decapeptides  
5 of CFA/I in one monkey. The monkey (2Z2) as immunized  
6 with three i.m. injections of CFA/I subunits in  
7 adjuvant, and its spleen cells were cultured with  
8 synthetic decapeptides which had been constructed using  
9 the Pepsan technique. The decapeptides represented  
10 the entire CFA/I protein. Values shown represent the  
11 proliferative response which occurred to 6.0  
12 micrograms/ml (Fig. 22), 0.6 micrograms/ml (Fig. 23),  
13 or 0.06 micrograms/ml (Fig. 24) of antigen  $\pm$  the  
14 standard deviation. The cpm of the control peptide was  
15  $1,553 \pm 33$  and the cpm of the media control was  $1,951 \pm$   
16 245.

17 Figure 25 shows that rabbits numbers 21 and  
18 22 received intraduodual administration of AF/R1  
19 microspheres at doses of AF/R1 of 200 micrograms (ug)  
20 on day 0 and 100 ug on day 7, 14, and 21 then  
21 sacrificed on day 31. The spleen, Peyer's patch and  
22 ileal lamina propria cells at  $6 \times 10^5$  in 0.2 ml in  
23 quadriplate were challenged with AF/RI and AF/R1 1-13,  
24 40-55, 79-94, 108-123, and 40-47, 79-85 synthetic  
25 peptides at 15, 1.5 and .15 ug/ml for 4 days. The  
26 supernatants were tested for IL-4 using the IL-4/IL-2

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1 dependent cell line cells CT4R at 50,000/well with 0.1  
2 ml of 6.25% supernatant for 3 days then pulsed with  
3 tritiated thymidine for 4 hrs, cells harvested and the  
4 tritiated thymidine incorporation determined, averaged  
5 and expressed with one standard deviation thousand  
6 counts per minute (kcpm).

7 Figure 26 shows that RDEC-1 colonization (log  
8 CFU/gm) in cecal fluids was similar in both groups  
9 (mean 6.3 vs 7.3;  $p=.09$ ).

10 Figure 27 shows that rabbits given AF/R1-MS  
11 remained well and 4/6 gained weight after challenge,  
12 whereas 9/9 unvaccinated rabbits lost weight after  
13 challenge (mean weight change +10 vs -270 grams  
14  $p<.001$ ).

15 Figure 28 shows that the mean score of RDEC-1  
16 attachment to the cecal epithelium was zero in  
17 vaccinated, and 2+ in unvaccinated animals.

18 Figure 29. Particle size distribution of  
19 CFA/II microsphere vaccine Lot L74F2 values are percent  
20 frequency of number or volume verses distribution.  
21 Particle size (diameter) in microns. 63% by volume are  
22 between 5-10 um and 88% by volume are less then 10 um.

23 Figure 30. Scanning electron photomicrograph  
24 of CFA/II microsphere vaccine Lot L7472 standard bar  
25 represents 5 um distance.

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1                   Figure 31. Twenty-two hour CFA/II release  
2                   study of CFA/II microsphere vaccine Lot L7472. Percent  
3                   cumulative release of CFA/II from three sample: A,  
4                   33.12 mgm; B, 29.50 mgm c, 24.20 mgm at 1, 3, 6, 8, 12  
5                   and 22 hour intervals. Average represents the mean  $\pm$   
6                   ISD.

7                   Figure 32. Serum IgG antibody reponse to  
8                   CFA/II microsphere vaccine Lot L7472 following 2 25 ug  
9                   protein IM immunization on day 0 in 2 rabbits.  
10                  Antibody determines on serial dilution of sera by ELISA  
11                  and expressed as mean titer versus day 0, 7 and 14.

12                  Figure 33. Serum IgG antibody response to  
13                  CFA/II microsphere vaccine Lot L7F2 following 2 25 ug  
14                  protein IM immunizations on day 0 if rabbit 107 & 109.  
15                  Antibody determined on serial dilution (in duplicate)  
16                  of sera by ELISA and expressed as mean titer versus day  
17                  0, 7 and 14.

18                  Figure 34. Lymphocyte proliferative  
19                  responses for Peyer's patch cells of rabbits 65 (figure  
20                  34 (a)), 66 (figure 34 (b)) , 83 (figure 34 (c)), 86  
21                  (figure 34 (d)), and 87 (figure 34 (e)) immunized  
22                  intraduodenally with 50 mgm protein of CFA/II  
23                  microsphere vaccine 4 and 7 days earlier. The cells  
24                  are challenged in vitro with CFA/II or BSA at 500, 50  
25                  and 5 ug/ml or media in triplicate. The uptake of  
26                  tritiated thymidine in Kcp is expressed as mean  $\pm$  ISD.

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1 Using the paired student t-test, the p values of 500  
2 ug/ml dose of CFA/II compared to media control are:  
3 65,  $p = 0.0002$ ; 66,  $p = 0.0002$ ; 83,  $p = 0.0002$ ; and 86,  $p$   
4  $= 0.0002$ .

5 Figure 35. Lymphocyte proliferative  
6 responses from Peyer's patch cells of rabbits 77  
7 (figure 35 (a)), 78 (figure 35 (b)), 80 (figure 35  
8 (c)), 88 (figure 35 (d)), and 91 (figure 35 (e))  
9 immunized intraduodenally with 50 mgm protein of CFA/II  
10 microspheres vaccine 14 and 7 days earlier. The cells  
11 are challenged in vitro with CFA with CFA/II or BSA at  
12 500, 50 and 5 ug/ml or media in triplicate the uptake  
13 of tritiated thymidine in  
14 Kcp is expressed as mean  $\pm$  ISD. Using the paired  
15 student t -test, the protein of 500 ug/ml dose of  
16 CFA/II compared to media control are: 77,  $p = 0.0001$ ;  
17 78;  $= 0.0015$ ; 80,  $p = \text{insignificant}$ ; 88,  $p = 0.0093$ ;  
18 and 91  $p = 0.0001$ .

19 Figure 36. ELISPOT assay of spleen cells  
20 from rabbits 65 (figure 36 (a)), 66 (figure 36 (b)), 83  
21 (figure 36 (c)), 86 (figure 36 (d)), and 87 (figure 36  
22 (e)) immunized intraduodenally with 50 mgm protein  
23 of CFA/II microsphere vaccine 14 and 7 days  
24 earlier. These were cells placed into microculture and  
25 tested on day 0, 1, 2, 3, 4 and 5 by ELISPOT for cells  
26 secreting antibodies specific for CFA/II antigen. The

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1 results are expressed as number per  $9 \times 10^6$  spleen  
2 cells versus culture day tested.

3 Figure 37. ELISPOT assay of spleen cells  
4 from normal control rabbits, 67, 69, 72 and 89. The  
5 cells were placed into microculture and tested on days  
6 0, 1, 2, 3, 4 and 5 by ELISPOT for cells secreting  
7 antibodies specific for CFA/II antigen. The results  
8 are expressed as number per  $9 \times 10^6$  spleen cells versus  
9 culture day tested.

10 Figure 38. Curve for determining vaccination  
11 dosages for regimen b.

12 Figure 39. Hepatitis B surface antigen  
13 release from 50:50 poly (DL-lactide-co-glycolide).

14 Figures 11 and 12 serve to illustrate that  
15 inclusion of Escherichia coli pilus antigen in  
16 microspheres enhances cellular immunogenicity.

17 A primary mucosal immune response,  
18 characterized by antipilus IgA, follows infection of  
19 rabbits with E. coli RDEC-1. However, induction of an  
20 optimal primary mucosal response by enteral vaccination  
21 with pilus antigen depends on immunogenicity of pilus  
22 protein, as well as such factors as its ability to  
23 survive gastrointestinal tract (GI) transit and to  
24 target immunoresponsive tissue. We tested the effect  
25 of incorporating AF/R1 pilus antigen into resorbable  
26 microspheres upon its ability to induce primary mucosal

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1 and systemic antibody responses after direct  
 2 inoculation into the GI tract. METHODS: rabbits were  
 3 inoculated with 50 micrograms of AF/R1 pilus antigen  
 4 alone or incorporated into uniformly sized (5-10  
 5 microns) resorbably microspheres (MIC) of  
 6 poly(DL-lactide-coglycolide). Inoculation was by  
 7 intra-duodenal (ID) intubation via endoscopy or  
 8 directly into the ileum near a Peyer's patch via the  
 9 RITARD procedure (with the cecum ligated to enhance  
 10 recovery of gut secretions and a reversible ileal tie  
 11 to slow antigen clearance). ID rabbits were sacrificed  
 12 at 2 weeks for collection of gut washes and serum.  
 13 RITARD rabbits were bled and purged weekly for 3 weeks  
 14 with Co-lyte to obtain gut secretions. Anti-pilus IgA  
 15 and IgG were measured by ELISA.

TABLE 1

17	<u>RESULTS</u> :*pos/test	RITARD-PILI	RITARD-MIC	ID-PILI
18	ID-MIC			
19	Anti-pilus IgA (fluid)	*7/8		4/8
20	1/2      0/3			
21	Anti-pilus IgG (serum)	0/8		3/8
22	0/2      1/3			

23 Native pilus antigen led to a mucosal IgA  
 24 resposne in 7/8 RITARD rabbits. MIC caused a similar  
 25 response in only 4/8, but the groups were not  
 26 statistically different. MIC (but not pili) induced



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1       some systemic IgG responses (highest in animals without  
2       mucosal responses). Results in rabbits inoculated ID  
3       were similar for pili, but no mucosal response to  
4       ID-MIC was noted. SUMMARY: Inoculation with pilus  
5       antigen produces a primary mucosal IgA response.  
6       Microencapsulation does not enhance this response,  
7       although the antigen remains immunogenic as shown by  
8       measurable mucosal and some strong serum responses. It  
9       must be determined whether priming with antigen in  
10      microspheres can enhance secondary responses.

#### 11                   B CELL EPITOPE DATA

#### 12                   Materials and Methods

13               **CFA/I PURIFICATION-** INTACT CFA/I pili were  
14       purified from H10407 (078:H-) as described by Hall et  
15       al, (1989) [20]. Briefly, bacteria grown on  
16       colonization factor antigen agar were subjected to  
17       shearing, with the shearate subjected to differential  
18       centrifugation and isopycnic banding on cesium chloride  
19       in the presence of N-lauryl sarkosine. CFA/I were  
20       dissociated to free subunits in 6M guanididinium HCl,  
21       0.2 M ammonium bicarbonate (2 hr, 25°), passed through  
22       an ultrafiltration membrane (Amicon XM 50 stirred cell,  
23       Danvers, MA), with concentration and buffer exchange to  
24       PBS on a YM 10 stirred cell (Amicon). Examination of  
25       dissociated pili by electron microscopy demonstrated a  
26       lack of pilus structure.

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1                   **Protein Sequencing-** The primary structure of  
2           CFA/I has been determined by protein sequencing  
3           techniques (Klemm, 1982) and through molecular cloning  
4           methods (Karjalainen, et al 1989) [21]. In these two  
5           studies there was agreement in all but two of the 147  
6           amino acid residues (at positions 53 and 74). To  
7           resolve the apparent discrepancies, CFA/I was  
8           enzymatically digested in order to obtain internal  
9           amino acid sequence. Trypsin or *S. aureus* V8 protease  
10          (sequencing grade, Boehringer Mannheim) was incubated  
11          with CFA/I at a 1:50 w:w ratio (Tris 50 mM, 0.1% SDS,  
12          pH 8.5 for 16h at 37° (trypsin) or 24°C (V8)). Digested  
13          material was loaded onto precast 16% tricine SDS-PAGE  
14          gels (Schagger and von Jagow, 1987) (Novex, Encinitis,  
15          CA) and run following manufacturers instructions.  
16          Separated samples were electrophoretically transferred  
17          to PVDF membranes (Westrans, Schleicher and Schuell,  
18          Keene, NH) following Matsiduria (1987) using the Novex  
19          miniblot apparatus. Blotted proteins were stained with  
20          Rapid Coomassie stain (Diversified Biotech, Newton  
21          Centre, MA). To obtain the desired fragment containing  
22          the residue of interest within a region accessible by  
23          automated gas phase sequencing techniques, molecular  
24          weights were estimated from standards of molecular  
25          weights 20,400 to 2,512 (trypsin inhibitor, myoglobin,  
26          and myoglobin cyanogen bromide fragments; Diversified

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1 Biotech) using the corrected molecular weights for the  
2 myoglobin fragments as given in Kratzin et al., (1989)  
3 [22]. The estimated molecular weights for the unknown  
4 CFA/I fragments were compared to calculated molecular  
5 weights of fragments as predicted for CFA/I from the  
6 sequence of CFA/I as analysed by the PEPTIDESORT  
7 program of a package developed by the University of  
8 Wisconsin Genetics Computer Group. Selected fragments  
9 were cut from the PVDF emembrane and subjected to gas  
10 phase sequencing (Applied Biosystem 470, Foster City,  
11 CA).

12 Monkey Immunization- Three rhesus monkeys (Macaca  
13 mulatta) were injected intramuscularly with 250 ug of  
14 dissociated CFA/I in complete Freund's adjuvant and  
15 subsequently with two injections of 250 ug of antigen in  
16 incomplete Freund's adjuvant at weekly intervals.  
17 Blood was drawn three weeks after primary immunization.

18 Peptide Synthesis- Continuous overlapping  
19 octapeptides spanning the entire sequence CFA/I were  
20 synthesized onto polyethylene pins by the method of  
21 Geysen et al. [16], also known as the PEPSCAN  
22 procedure. Derivitized pins and software were  
23 purchased from Cambridge Research Biochemicals (Valley  
24 Stream, NY). Fmoc-amino acid pentafluorophenyl esters  
25 were purchased from Peninsular Laboratories (Belmont,  
26 CA), 1-hydroxybenzotriazole monohydrate (HYBT) was

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1 purchased from Aldrich, and reagent grade solvents from  
2 Fisher. To span the entire sequence of CFA/I with a  
3 single amino acid overlap of from one peptide to the  
4 next, 140 total pins were necessary, with a second  
5 complete set of 140 pins synthesized simultaneously.

6 ELISA procedure- Sera raised in monkeys to  
7 purified dissociated pili were incubated with the pins  
8 in the capture ELISA assay of Geysen et al., [16] with  
9 the preimmune sera of the same animal tested at the  
10 same dilution simultaneously with the duplicate set of  
11 pins. Dilution of sera used on the pins was chosen by  
12 initial titration of sera by standard ELISA assay and  
13 immunodot blot assay against the same antigen.

#### 14 RESULTS

15 It was essential to utilize the correct sequence  
16 of CFA/I in the synthesis of the pins for both T- and  
17 B-cell experiments to carry out the studies as planned.  
18 At issue were the amino acids at position 53 and 74;  
19 incorrect residues at those positions would effect 36  
20 of 138 pins (26%) for T-cell epitope analysis and 30 of  
21 140 pins (21%) for B-cell analysis. To resolve the  
22 discrepancy in the literature, purified CFA/I was  
23 proteolytically digested separately with trypsin and  
24 with *S. aureus* V8 protease (V8). These enzymes were  
25 chosen in order to give fragments with the residues of  
26 interest (53 and 74) relatively near to the N-terminus

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1 for automated Edman degradation (preferably 1-15  
2 residues). These digests were separated on tricine  
3 SDS-PAGE gels (Fig. 16A) and molecular masses of  
4 fragments estimated. A fragment of 3459 calculated  
5 molecular mass is expected from the trypsin digest  
6 (corresponding to amino acids 62-94) and a fragment of  
7 5889 calculated molecular mass is expected from the V8  
8 digest (residues 42-95). These fragments were located  
9 within each digest (arrows in Fig. 16), and a companion  
10 gel with four lanes of each digest was run,  
11 electrophoretically transferred to PVDF, the bands  
12 excised and sequenced. N-terminal sequences of each  
13 fragment are given in Fig. 16B. The N-terminal  
14 eighteen residues from the trypsin fragment were  
15 determined that corresponded to positions 62-79 in  
16 CFA/I. Position 74, a serine residue was consistent  
17 with that determined by Karjalainen et al.,  
18 (Karjalainen et al., 1989). Nineteen residues of the  
19 V8 fragment were determined, corresponding to residues  
20 41-60 of the parent protein. The twelfth residue of the  
21 fragment contained an aspartic acid, also consistent  
22 with Karjalainen et al., (1989). All other residues  
23 sequenced were consistent with those published  
24 previously (including residues 1-29, not shown). For  
25 the following peptide synthesis were therefore

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1 utilized the complete amino acid sequence of CFA/I  
2 consistent with Karjalainen et al., (1989).

3 Sera from monkeys immunized with CFA/I subunits  
4 were tested in a modified ELISA assay, with the  
5 preimmunization sera tested simultaneously with  
6 duplicate pins. Assays results are displayed in Fig.  
7 17. Monkey 2Z2 (fig. 2A) responded strongly to six  
8 regions of the CFA/I sequence. Peptide 14 (the  
9 octapeptide 14-21) gave the strongest response with  
10 four pins adjacent to it (11, 12, 13, and 15) also  
11 appearing to bind significant antibody. The other 2Z2  
12 epitopes are centered at peptides 3, 22, 33, 93, and  
13 124. Monkey 184D (Fig. 17B) also responded strongly to  
14 peptide 14, although the maximum response was to  
15 peptide 13, with strong involvement of peptide 12 in  
16 the epitope. Additional epitopes recognized by 184D  
17 were centered at peptides 22, 33, 66, and 93. The  
18 third monkey serum tested, 34, responded to this region  
19 of the CFA/I primary structure, both at peptides 1, 12  
20 and weakly at 14. Two other epitopes were identified  
21 by 34, centered at peptides 67 and 128. Figure 18  
22 illustrates the amino acids corresponding to the  
23 epitopes of CFA/I as defined by the response of these  
24 three monkeys aligned with the entire primary  
25 structure. The entire antigenic determinants are  
26 mapped and areas of overlap with other epitopes

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1 (consensus sites) are displayed. These epitopes are  
2 summarized in Table 1.

3 T Cell Epitope Data

4 Materials and Methods

5 Animals. Three healthy adult Macaca mulatta  
6 (Rehesus) monkeys (approximately 7 kg each) were used  
7 in this study. Their medical records were examined to  
8 assure that they had not been in a previous protocol  
9 which would preclude their use in this study. Each  
10 monkey was sedated with ketamine HCL1 at standard  
11 dosage and blood was drawn to obtain preimmune serum.

12 Antigen. CFA/I pili were purified from E. coli  
13 strain H107407 (serotype 078:H11) by ammonium sulfate  
14 precipitation using the method of Isaacson [17]. The  
15 final preparation migrated as a single band on  
16 SD-polyacrylamide gel electrophoresis and was shown to  
17 be greater than 95% pure by scanning with laser  
18 desitometry when stained with coomassie blue. The pili  
19 were then dissociated into CFA/I pilin subunits.

20 Immunization. Each monkey was given 25 mg of  
21 purified CFA/I pilin subunits, which had been  
22 emulsified in Complete Freund's Adjuvant, by single  
23 i.m. injection (0.5 ml). For each animal, the initial  
24 dose of antigen was followed by two similar injections  
25 in Incomplete Freund's Adjuvant at seven day intervals.

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1           Peptide Antigens. The peptides were synthesized  
2           based on the published sequence of CFA/I [18] using the  
3           Geysen pin method (Pepscan procedure) [16] with  
4           equipment and software purchased from Cambridge  
5           Research Biochemicals, Inc. (Wilmington, DE).  
6           Fmoc-amino acid pentafluorophenyl esters were purchased  
7           from Peninsula Laboratories (Belmont, CA) and used  
8           without further treatment or analysis. The activating  
9           agent 1-hydroxybenzotriazole monohydrate (HOBT) was  
10          purchased from Aldrich Chemical Company (Milwaukee,  
11          WI). Solvents were reagent grade from Fisher Scientific  
12          (Springfield, NJ).

13               Two schemes were used to synthesize the peptides.  
14          Peptides for the B-cell tests were synthesized as  
15          octamers and remained linked to the resin. However,  
16          the peptides used to search for T-cell epitopes were  
17          synthesized as decamers with an additional Asp-Pro  
18          spacer between the pins and the peptides of interest.  
19          The Asp-Pro linkage is acid labile allowing cleavage of  
20          the decamers from the pins for T-cell proliferation  
21          assays [15]. The peptides were cleaved in 70% formic  
22          acid for 72 hours at 37 degrees C. The acid solution  
23          was removed by evaporation (Speed-Vac, Savant  
24          Instruments, Framingdale, NY) followed by rehydration  
25          with distilled deionized water and lyophilization. The  
26          resulting cleaved peptides were used without further



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1 treatment or analysis. The yield was approximately 10  
2 ug per pin, approximately 10 per cent on a molar basis  
3 of the total amount of proline on each pin as  
4 determined by quantitative amino acid analysis.

5 Residues 12 and 13 on the CFA-1 protein are Asp  
6 and Pro, respectively, the same sequence used to cleave  
7 the peptides from the pins. Therefore, to prevent  
8 truncated peptides from the native sequence during the  
9 cleavage process, two substitutions were made for  
10 Asp-12. One substitution was a glutamic acid residue  
11 for the aspartic acid, a substitution to retain the  
12 carboxylic acid functional group. The second  
13 substitution was an asparagine residue to conserve the  
14 approximate size of the side chain while retaining some  
15 hydrophilicity. Each substitution was tested in the  
16 T-cell proliferation assay. Both substitutions as well  
17 as the native sequence were analyzed by ELISA. For  
18 both the T cell and B cell assays, additional sequences  
19 not found on the protein were synthesied and used as  
20 control peptides.

21 Lymphocyte proliferation. At day 10-14 following  
22 the final inoculation of antigen, the monkeys were  
23 again sedated with ketamine HCl, and 50 ml of blood was  
24 drawn from the femoral artery for serum preparation.  
25 Animals were then euthanized with an overdose of  
26 pentothal and spleen was removed. Single cell

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1 suspensions were prepared and washed in Dulbecco's  
2 modified Eagle medium (Gibco Laboratories, Grand  
3 Island, NY) which had been supplemented with penicillin  
4 (100 units/ml), streptomycin (100 ug/ml), L-glutamine  
5 (2mM), and HEPES Buffer (10 mM) all obtained from Gibco  
6 Laboratories, as well as MEM non-essential amino acid  
7 solution (0.1 mM), MEM [50x] amino acids (2%), sodium  
8 bicarbonate (0.06%), and  $5 \times 10^{-5}$  M 2-ME all obtained  
9 from Sigma Chemical Company (St. Louis, MO) [cDMEM].  
10 Erythrocytes in the spleen cell suspension were lysed  
11 using standard procedures in an ammonium chloride  
12 lysing buffer. Cell suspensions were adjusted to  $10^7$   
13 cells per ml in cDMEM, and autologous serum was added to  
14 yield a final concentration of 1.0%. Cells (0.05 ml)  
15 were plated in 96-well flat bottom culture plates  
16 (Costar, Cambridge, MA) along with 0.05 ml of various  
17 dilutions of antigen in cDMEM without serum (yielding a  
18 0.5% final concentration of autologous serum) and were  
19 incubated at 37 degrees C in 5% CO<sub>2</sub>. Each peptide was  
20 tested at 6.0, 0.6, 0.06 ug/ml. All cultures were  
21 pulsed with 1 uci [<sup>3</sup>H]thymidine (25 Ci/mmol, Amersham,  
22 Arlington Heights, IL) on day 4 of culture and were  
23 harvested for scintillation counting 6 hours later.

## ELISA.

25 Epitope prediction. Software designed to predict  
26 B cell epitopes based on hydrophilicity, flexibility,

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1 and other criteria was developed by the University of  
2 Wisconsin Genetics Computer Group [19]. Software  
3 designed to predict T cell epitopes based on the  
4 Rothbard method [7] was written by Stephen Van Albert  
5 (The Walter Reed Army Institute of Research,  
6 Washington, D.C.). Software designed to predict T cell  
7 epitopes based on the Berzofsky method was published as  
8 the AMPHI program [9]. It predicts amphipathic amino  
9 acid segments by evaluating 7 or 11 residues as a block  
10 and assigning the score to the middle residue of that  
11 block.

12 Statistics. All lymphocyte proliferations were  
13 conducted in replicates of four, and standard  
14 deviations of the counts per minute (cpm) are shown.  
15 Statistical significance (p value) for the  
16 proliferative assay was determined using the Student's  
17 t test to compare the cpm of quadruplicate wells  
18 cultured with the CFA/I peptides to the cpm of wells  
19 cultured with a control peptide.

## 20 RESULTS

21 Prediction of T cell epitopes within the CFA/I  
22 molecule. To identify possible T cell epitopes within  
23 the CFA/I molecule, amphipathic amino acid segments  
24 were predicted by evaluating 7 or 11 residues as a  
25 block using the AMPHI program [9]. Possible t cell  
26 epitopes were also identified using criteria published

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1 by Rothbard and Taylor [7]. The sequence numbers of  
2 the first amino acid of the predicted segments are  
3 shown in Table 1.

4 Lymphocyte proliferation of monkey spleen cells  
5 to CFA/I synthetic peptides. To determine which  
6 segments of the CFA/I protein are able to stimulate  
7 proliferation of CFA/I immune primate lymphocytes in  
8 vitro, three Rhesus monkeys were immunized with CFA/I  
9 subunits, and their splenic lymphocytes were cultured  
10 with synthetic overlapping decapeptides which  
11 represented the entire CF/I sequence. Concentrations  
12 of peptides used as antigen were 6.0, 0.6, and 0.6  
13 ug/ml. Proliferative responses to the decapeptides  
14 were observed in each of the three monkeys (fig. 1-3).  
15 The majority of the responses occurred at the 0.6 and  
16 0.06 ug/ml concentrations of antigen and within  
17 distinct regions of the protein (peptides beginning  
18 with residues 8-40, 70-80, and 27-137). A comparison  
19 of the responses at the 6.0, 0.6 and 0.06 ug/ml  
20 concentrations antigenic peptide for one monkey (2&2)  
21 are shown (fig. 4-6). Taking into account all  
22 concentrations of antigen tested, spleen cells from  
23 monkey 184D demonstrated a statistically significant  
24 response to decapeptides beginning with CFA/I amino  
25 acid residues 3, 4, 8, 12, 15, 21, 26, 28, 33, 88, 102,  
26 10, 133, 134, and 136 (fig. 19). Monkey 34 had a

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1 significant response to decapeptides beginning with  
2 residues 24, 31, 40, 48, 71, 72, 77, 78, 80, 87, and  
3 102, 126 and 133 (Fig. 20); monkey 222 responded to  
4 decapeptides which began with residues 4, 9, 11, 12,  
5 13, 14, 15, 16, 17, 20, 27, 35, 73, 79, 18, 127, 129,  
6 132, and 133 (fig. 19). Peptides beginning with amino  
7 acid residues 3 through 2 were synthesized with either  
8 a glutamic acid or an asparagine substituted for the  
9 aspartic acid residue at position twelve to prevent  
10 truncated peptides. The observed responses to peptides  
11 beginning with residue 8 (monkey 184d), and residues 9,  
12 11, 12 (monkey 222) occurred in response to peptides  
13 that had the glutamic acid substitution. However, the  
14 observed responses to peptides beginning with residue  
15 3, 4, and 12 (monkey 184D), as well as residue 4 (monkey  
16 222) occurred in response to peptides that had the  
17 asparagine substitution. Monkey 34 did not respond to  
18 any of the peptides that had the substitution at  
19 position twelve. All other responses shown were to the  
20 natural amino acid sequence of the CFA/I protein.  
21 Statistical significance was determined by comparing  
22 the cpm of quadruplicate wells cultured with the CFA/I  
23 peptides to the cpm of wells cultured with the CFA/I  
24 peptides to the cpm of wells cultured with a control  
25 peptide.

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1           Analysis of decapeptides that supported  
2           proliferation of lymphocytes from CFA/I immune animals.  
3           Of the 39 different peptides that supported  
4           proliferative responses, thirty contained a serine  
5           residue, 19 contained a serine at either position 2, 3,  
6           or 4, and nine had a serine specifically at position 3.  
7           Some of the most robust responses were to the peptides  
8           that contained a serine residue at the third position.  
9           The amino acid sequence of four such peptides is shown  
10          in Table 3.

#### 11           VII. DETAILED DESCRIPTION OF THE INVENTION

12          Applicants have discovered efficacious  
13          pharmaceutical compositions wherein the relative  
14          amounts of antigen to the polymeric matrix are within  
15          the ranges of 0.1 to 1.5% antigen (core loading) and  
16          99.9 to 98.5% polymer, respectively. It is preferred  
17          that the relative ratio between the lactide and  
18          glycolide component of the  
19          poly(DL-lactide-co-glycolide) (DL-PLG) is within the  
20          range of 40:60 to 0:100. However, it is understood  
21          that effective core loads for certain antigens will be  
22          influenced by its microscopic form (i.e. bacteria,  
23          protozoa, viruses or fungi) and type of infection being  
24          prevented. From a biological perspective, the DL-PLG  
25          or glycolide monomer excipient are well suited for in  
26          vitro drug (antigen) release because they elicit a

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1       minimal inflammatory response, are biologically  
2       compatible, and degrades under physiologic conditions  
3       to products that are nontoxic and readily metabolized.

4               Surprisingly, applicants have discovered an  
5       extremely effective method for the protection against  
6       bacterial or viral infections in the tissue of a mammal  
7       (human or nonhuman animal) caused by enteropathogenic  
8       organisms comprising administering orally to said  
9       animal an immunogenic amount of a pharmaceutical  
10      composition consisting essentially of an antigen  
11      encapsulated within a biodegradable polymeric matrix.  
12      When the polymeric matrix is DL-PLG, the most preferred  
13      relative ratio between the lactide and glycolide  
14      component is within the range of 48:52 to 58:42. The  
15      bacterial infection can be caused by bacteria  
16      (including any derivative thereof) which include  
17      Salmonella typhi, Shigella sonnei, Shigella flexneri,  
18      Shigella dysenteriae, Shigella boydii, Escheria coli,  
19      Vibro cholera, yersinia, staphylococcus, clostridium  
20      and campylobacter. Representative viruses contemplated  
21      within the scope of this invention, susceptible to  
22      treatment with the above-described pharmaceutical  
23      compositions, are quite extensive. For purposes of  
24      illustration, a partial listing of these viruses  
25      (including any derivative thereof) include hepatitis A,  
26      hepatitus B, rotaviruses, polio virus human

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1 immunodeficiency viruses (HIV), Herpes Simplex virus  
2 type 1 (cold sores), Herpes Simplex virus type 2  
3 (Herpesvirus genitalis), Varicella-zoster virus  
4 (chicken pox, shingles), Epstein-Barr virus (infectious  
5 mononucleosis; glandular fever; and Burkittis  
6 lymphoma), and cytomegalo viruses.

7 A further representation description of the  
8 instant invention is as follows:

9 A. (1) To homogeneously disperse antigens of  
10 enteropathic organisms within the polymeric matrix of  
11 biocompatible and biodegradable microspheres, 1  
12 nanogram (ng) to 12 microns in diameter, utilizing  
13 equal molar parts of polymerized lactide and glycolide  
14 (50:50 DL-PLG, i.e. 48:52 to 58:42 DL-PLG) such that  
15 the core load is within the range of about 0.1 to 1.5%  
16 by volume. The microspheres containing the dispersed  
17 antigen can then be used to immunize the intestine to  
18 produce a humoral immune response composed of secretory  
19 antibody, serum antibody and a cellular immune response  
20 consisting of specific T-cells and B-cells. The immune  
21 response is directed against the dispersed antigen and  
22 will give protective immunity against the pathogenic  
23 organism from which the antigen was derived.

24 (2) AF/R1 pilus protein is an adherence  
25 factor that allows E. coli RDEC-1 to attach to rabbit  
26 intestinal brush borders thus promoting colonization



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1       resulting in diarrhea. AF/R1 pilus protein was  
2       homogeneously dispersed within a polymeric matrix of  
3       biocompatible and biodegradable microspheres, 1-12  
4       microns in diameter (Figure 1 and photograph 1) using  
5       equal molar parts of polymerized lactide and glycolide  
6       (50:50 DL-PLG) such that the core load was .62% by  
7       weight.

8                   (3) The microspheres were found to contain  
9       immunogenic AF/R1 by immunizing both rabbit spleen  
10      (Figure 2) and Peyer's patch (Figure 3) B-cells in  
11      vitro. The resultant cell supernatants contained  
12      specific IgM antibody which recognized the AF/R1. The  
13      antibody response was comparable to immunizing with  
14      AF/R1 alone.

15                   (4) Microspheres containing 50  
16      micrograms of AF/R1 were used to intrainstestinally  
17      (intraduodenally) immunize rabbits on two separate  
18      occasions 1 week apart. One week later, compared to  
19      rabbits receiving AF/R1 alone, the intestinal lymphoid  
20      tissue, Peyer's patches, demonstrated an enhanced  
21      cellular immune response to AF/R1 and to three AF/R1  
22      linear peptide fragments 40-55, 79-94 and 108-123 by  
23      both lymphocyte transformation (T-cells) (Figures 4 and  
24      5) and antibody producing B-cells (Figures 6 and 7).  
25      Similarly enhanced B-cell responses were also detected  
26      in the spleen (Figures 8 and 9). An enhanced T-cell

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1 response was also detected in the mesenteric lymph node  
2 and the spleen to one AF/R1 peptide fragment, 40-55  
3 (Figures 10 and 11). The cellular immune response at  
4 two weeks was too early for either a serum or secretory  
5 antibody response (See Results in Table 1); but  
6 indicates that a secretory antibody response will  
7 develop such that the rabbits so immunized could be  
8 protected upon challenge with the E. coli RDEC-1.

9 B. Microspheres do not have to be made up just  
10 prior to use as with liposomes. Also liposomes have  
11 not been effective in rabbits for intestinal  
12 immunization of lipopolysaccharide antigens.

13 C. (1) Only a small amount of antigen is  
14 required (ugs) when dispersed within microspheres  
15 compared to larger amounts (mgms) when antigen is used  
16 alone for intestinal immunization.

17 (2) Antigen dispersed within  
18 microspheres can be used orally for intestinal  
19 immunization whereas antigen alone used orally even  
20 with gastric acid neutralization requires a large  
21 amount of antigen and may not be effective for  
22 intestinal immunization.

23 (3) Synthetic peptides with and without  
24 attached synthetic adjuvants representing peptide  
25 fragments of protein antigens can also be dispersed  
26 within microspheres for oral-intestinal immunization.

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1 Free peptides would be destroyed by digestive processes  
2 at the level of the stomach and intestine. Any  
3 surviving peptide would probably not be taken up by the  
4 intestine and therefore be ineffective for intestinal  
5 immunization.

6 (4) Microspheres containing antigen maybe  
7 placed into gelatin-like capsules for oral  
8 administration and intestinal release for improved  
9 intestinal immunization.

10 (5) Microspheres promote antigen uptake  
11 from the intestine and the development of cellular  
12 immune (T-cell and B-Cell) responses to antigen  
13 components such as linear peptide fragments of protein  
14 antigens.

15 (6) The development of intestinal T-cell  
16 responses to antigens dispersed within microspheres  
17 indicate that T-cell immunological memory will be  
18 established leading to long-lived intestinal immunity.  
19 This long-lived intestinal immunity (T-cell) is very  
20 difficult to establish by previous means of intestinal  
21 immunization. Failure to establish long-lived  
22 intestinal immunity is a fundamental difficulty for  
23 intestinal immunization with non-viable antigens.  
24 Without intestinal long-lived immunity only a short  
25 lived secretory antibody response is established

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1       lasting a few weeks after which no significant  
2       immunological protection may remain.

3               D.       (1) Oral intestinal immunization of  
4       rabbits against E. coli RDEC-1 infection using either  
5       whole killed organisms, pilus protein preparations or  
6       lipopolysaccharide preparations.

7                       (2) Microspheres containing adherence pilus  
8       protein AF/R1 or its antigen peptides for oral  
9       intestinal immunization of rabbits against RDEC-1  
10      infection.

11                      (3) Oral-intestinal immunization of humans  
12      against enterotoxigenic E. coli infection using either  
13      whole killed organisms, pilus protein preparations or  
14      lipopolysaccharide preparations.

15                      (4) Microspheres containing adherence pilus  
16      proteins CFA/I, II, III and IV or their antigen  
17      peptides for oral intestinal immunization of humans  
18      against human enterotoxigenic E. coli infections.

19                      (5) Oral-intestinal immunization of humans  
20      against other enteric pathogens as salmonella,  
21      shigella, camphlobacter, hepatitis-A virus, rota virus  
22      and polio virus.

23                      (6) Oral-intestinal immunization of animals  
24      and humans for mucosal immunological protection at  
25      distal mucosal sites as the bronchial tree in lungs,  
26      genito-urinary tract and breast tissue.

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1                   E. (1) The biocompatible, biodegradable  
2 co-polymer has a long history of being safe for use in  
3 humans since it is the same one used in resorbable  
4 suture material.

5                   (2) By using the microspheres, we are now  
6 able to immunize the intestine of animals and man with  
7 antigens not normally immunogenic for the intestinal  
8 mucosa because they are either destroyed in the  
9 intestine, unable to be taken up by the intestinal  
10 mucosa or only weakly immunogenic if taken up.

11                   (3) Establishing long-lived immunological  
12 memory in the intestine is now possible because T-cells  
13 are immunized using microspheres.

14                   (4) Antigens that can be dispersed into  
15 microspheres for intestinal immunization include the  
16 following: proteins, glycoproteins, synthetic  
17 peptides, carbohydrates, synthetic polysaccharides,  
18 lipids, glycolipids, lipopolysaccharides (LPS),  
19 synthetic lipopolysaccharides and with and without  
20 attached adjuvants such as synthetic muramyl dipeptide  
21 derivatives.

22                   (5) The subsequent immune response can be  
23 directed to either systemic (spleen and serum antibody)  
24 or local (intestine, Peyer's patch) by the size of the  
25 microspheres used for the intestinal immunization.  
26 Microspheres 5-10 microns in diameter remain within

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1       macrophage cells at the level of the Peyer's patch in  
2       the intestine and lead to a local intestinal immune  
3       response. Microspheres 1 ng - 5 microns in diameter  
4       leave the Peyer's patch contained within macrophages  
5       and migrate to the mesenteric lymph node and to the  
6       spleen resulting in a systemic (serum antibody) immune  
7       response.

8                       (6) Local or systemic antibody mediated  
9       adverse reactions because of preexisting antibody  
10      especially cytophyllic or IgE antibody may be minimized  
11      or eliminated by using microspheres because of their  
12      being phagocytized by macrophages and the antigen is  
13      only available as being attached to the cell surface  
14      and not free. Only the free antigen could become  
15      attached to specific IgE antibody bound to the surface  
16      of mast cells resulting in mast cell release of  
17      bioactive amines necessary for either local or systemic  
18      anaphylaxis.

19                      (7) Immunization with microspheres  
20      containing antigen leads to primarily IgA and IgG  
21      antibody responses rather than an IgE antibody  
22      response, thus preventing subsequent adverse IgE  
23      antibody reactions upon reexposure to the antigen.

24                      In addition to the above, the encapsulation of  
25      the following synthetic peptides are contemplated and

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1 considered to be well within the scope of this  
2 invention:

- 3 (1) AF/R1 40-55;  
4 (2) AF/R1 79-94;  
5 (3) AF/R1 108-123;  
6 (4) AF/R1 1-13;  
7 (5) AF/R1 pepscan 16AA;  
8 (6) CFA/I 1-13; and  
9 (7) CFA/I pepscan 16AA.  
10 (8) Synthetic Peptides Containing CFA/I Pilus

11 Protein

12 T-cell Epitopes (Starting Sequence #  
13 given)

14

15 4(Asn-Ile-Thr-Val-Thr-Ala-Ser-Val-Asp-Pro),

16

17 8(Thr-Ala-Ser-Val-Asp-Pro-Val-Ile-Asp-Leu),

18

19 12(Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),

20

21 15(Ile-Asp-Leu-Leu-Gln-Ala-Asp-Gly-Asn-Ala),

22

23 20(Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),

24

25 26(Pro-Ser-Ala-Val-Lys-Leu-Ala-Tyr-Ser-Pro),

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1  
2 72 (Leu-Asn-Ser-Thr-Val-Gln-Met-Pro-Ile-Ser),  
3  
4 78 (Met-Pro-Ile-Ser-Val-Ser-Trp-Gly-Gly-Gln),  
5  
6 87 (Gln-Val-Leu-Ser-Thr-Thr-Ala-Lys-Glu-Phe),  
7  
8 126 (Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr), and  
9  
10 133 (Gly-Asn-Tyr-Ser-Gly-Val-Val-Ser-Leu-Val), and  
11 mixtures thereof.

12 (9) Synthetic Peptides Containing CFA/I Pilus  
13 Protein B-cell (antibody) Eptiopes (Starting Sequence #  
14 given)

15 3 (Lys-Ana-Ile-Thr-Val-Thr-Ala-Ser-Val),  
  
16 11 (Val-Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),  
17 22 (Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),  
  
18 32 (Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe-Lys-Thr-Phe-  
19 Glu-Ser-Tyr-Arg-Val),  
20 32 (Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe),  
21 38 (Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val),  
22 66 (Pro-Gln-Leu-Thr-Asp-Val-Leu-Asn-Ser),  
23 93 (Ala-Lys-Glu-Phe-Glu-Ala-Ala-Ala),  
24 124 (Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr),



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1 127 (Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser),

2 and

3 124 (Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-

4 Ser), and mixtures thereof.

5 (10) synthetic peptides containing CFA/I  
6 pilus protein T-cell and B-cell (antibody) epitopes  
7 (Starting Sequence # given)

8 3(Lys-Asn-Ile-Thr-Val-Thr-Ala-Ser-Bal-Asp-Pro),

9 8 (Thr-Ala-Ser-Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-Ala-A  
10 sp) ,

11 11 (Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-Ala-Asp),

12 20 (Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val) ,

13           124 (Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser  
14           ), and

15 126(Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and  
16 mixtures thereof.

17           (11) synthetic peptides containing CFA/I pilus  
18           protein T-cell and B-cell (antibody) epitopes (Starting  
19           Sequence # given)

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1                    CFA/I pilus protein T-cell epitopes

2

3                    4 (Asn-Ile-Thr-Val-Thr-Ala-Ser-Val-Asp-Pro) ,

4

5                    8 (Thr-Ala-Ser-Val-Asp-Pro-Val-Ile-Asp-Leu) ,

6

7                    12 (Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp) ,

8

9                    15 (Ile-Asp-Leu-Leu-Gln-Ala-Asp-Gly-Asn-Ala) ,

10

11                    20 (Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val) ,

12

13                    26 (Pro-Ser-Ala-Val-Lys-Leu-Ala-Tyr-Ser-Pro) ,

14

15                    72 (Leu-Asn-Ser-Thr-Val-Gln-Met-Pro-Ile-Ser) ,

16

17                    78 (Met-Pro-Ile-Ser-Val-Ser-Trp-Gly-Gly-Gln) ,

18

19                    87 (Gln-Val-Leu-Ser-Thr-Thr-Ala-Lys-Glu-Phe) ,

20

21                    126 (Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr) , and

22

23                    133 (Gly-Asn-Tyr-Ser-Gly-Val-Val-Ser-Leu-Val) ; and

24                    synthetic peptides containing CFA/I pilus protein

25                    B-cell (antibody) epitopes (Starting Sequence # given)

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- 1                   CFA/I pilus protein B-cell epitopes  
2                   3 (Lys-Ana-Ile-Thr-Val-Thr-Ala-Ser-Val) ,  
  
3                   11 (Val-Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp) ,  
4                   22 (Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val) ,  
  
5                   32 (Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe-Lys-Thr-Phe-  
6                                   Glu-Ser-Tyr-Arg-Val) ,  
7                   32 (Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe) ,  
8                   38 (Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val) ,  
9                   66 (Pro-Gln-Leu-Thr-Asp-Val-Leu-Asn-Ser) ,  
10                  93 (Ala-Lys-Glu-Phe-Glu-Ala-Ala-Ala) ,  
11                  124 (Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr) ,  
12                  127 (Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser) ,  
13                  and  
  
14                  124 (Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-  
15                                   Ser) , and mixtures thereof.  
  
16                  (12) synthetic peptides containing CFA/I pilus  
17                  protein T-cell and B-cell (antibody) epitopes (Starting  
18                  Sequence # given)  
  
19                  CFA/I pilus protein T-cell epitopes  
20                  3 (Lys-Ana-Ile-Thr-Val-Thr-Ala-Ser-Val) ,

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1        11 (Val-Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),  
2                22 (Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),  
  
3        32 (Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe-Lys-Thr-Phe-  
4                Glu-Ser-Tyr-Arg-Val),  
5                32 (Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe),  
6                38 (Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val),  
7                66 (Pro-Gln-Leu-Thr-Asp-Val-Leu-Asn-Ser),  
8                93 (Ala-Lys-Glu-Phe-Glu-Ala-Ala-Ala),  
9                124 (Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr),  
10               127 (Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser),  
11        and  
  
12        124 (Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-  
13               Ser); and  
14        synthetic peptides containing CFA/I pilus protein  
15        T-cell and B-cell (antibody) epitopes (Starting  
16        Sequence # given)  
  
17                CFA/I pilus protein B-cell epitopes  
  
18        3 (Lys-Asn-Ile-Thr-Val-Thr-Ala-Ser-Bal-Asp-Pro),  
  
19        8 (Thr-Ala-Ser-Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-  
20               Ala-Asp),

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1        11(Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-Ala-Asp),  
2                    20(Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),  
  
3        124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser  
4        ), and  
  
5        126(Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and  
6        mixtures thereof.

7                We contemplate that the peptides can be used in  
8        vaccine constructed for systemic administration.

9                    VIII. EXAMPLES

10                The peptides in (8), (9), and (10) above can be  
11        made by classical solution phase synthesis, solid phase  
12        synthesis or recombinant DNA technology. These  
13        peptides can be incorporated in an oral vaccine to  
14        prevent infection by CFA/I bearing enteropathogenic E.  
15        coli.

16                The herein offered examples provide methods for  
17        illustrating, without any implied limitation, the  
18        practice of this invention in the prevention of  
19        diseases caused by enteropathogenic organisms.

20                The profile of the representative experiments  
21        have been chosen to illustrate the effectiveness of the  
22        immunogenic polymeric matrix-antigen composites.

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1 All temperatures not otherwise indicated are in  
2 degrees Celcius (°C) and parts or percentages are given  
3 by weight.

4 IX. MATERIALS AND METHODS

5 Animals. New Zealand White male rabbits were  
6 purchased from Hazelton Research Products (Denver, PA),  
7 and were shown to be free of current RDEC-1 infection  
8 by culture of rectal swabs. Animals were 1-2 kg of body  
9 weight and lacked agglutinating anti-AF/R1 serum  
10 antibody at the time of the study.

11 Antigens. AF/R1 pili from E. coli RDEC-1 (015:H:K  
12 non-typable) were purified by an ammonium sulfate  
13 precipitation method. The final preparation migrated  
14 as a single band on SDS-polyacrylamide gel  
15 electrophoresis and was shown to be greater than 95%  
16 pure by scanning with laser densitometry when stained  
17 with coomassie blue. Briefly, equal molar parts of  
18 DL-lactide and glycolide were polymerized and then  
19 dissolved to incorporate AF/R1 into spherical  
20 particles. The microspheres contained 0.62% protein by  
21 weight and ranged in size from 1 to 12 micrometers.  
22 Both the microencapsulated and non-encapsulated AF/R1  
23 were sterilized by gamma irradiation (0.3 megarads)  
24 before use.

25 Synthetic peptides (16 amino acids each) were  
26 selected by theoretical criteria from the amino acid

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1 sequence of AF/R1 as deduced from the nucleotide  
2 sequence. Three sets of software were used for the  
3 selections. Software designed to predict B cell  
4 epitopes based on hydrophilicity, flexibility, and  
5 other criteria was developed by the University of  
6 Wisconsin Genetics Computer Group. Software designed  
7 to predict T cell epitopes was based on the Rothbard  
8 method was written by Stephen Van Albert (The Walter  
9 Reed Army Institute of Research, Washington, D.C.).  
10 Software designed to predict T cell epitopes based on  
11 the Berzofsky method is published as the AMPHI program.  
12 The selected peptides were synthesized by using  
13 conventional Merrifield solid phase technology. AF/R1  
14 40-55 (Thr-Asn-Ala-Cly-Thr-Asp-Ile-Gly-  
15 Ala-Asn-Lys-Ser-Phe-Thr-Leu-Lys) was chosen as a  
16 probable B cell epitope for two reasons: (a) due to its  
17 high hydrophilic and flexibility indices, and (b)  
18 because it was not predicted to be a T cell epitope by  
19 either the Rothbard or Berzofsky method. AF/R1 79-94  
20 (Val-Asn-Gly-Ile-Gly-Asn-Leu-Ser-Gly-Lys-Ala-Ile-Asp-Al  
21 a- His-Val) was selected as a probable T cell eptiope  
22 because it contained areas predicted as a T cell  
23 epitope by both methods and because of its relatively  
24 low hydrophilic and flexibility indices. AF/R1 108-123  
25 (Asp-Thr-Asn-Ala-Asp-Lys-  
26 Glu-Ile-Lys-Ala-Gly-Gin-Asn-Thr-Val-Asp) was selected

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1 as both a T and B cell epitope. AF/R1 40/47/79-86 was  
2 produced in continuous synthesis  
3 (Thr-Asn-Ala-Cly-Thr-Asp-Ile-Gly-Val-Asn-GlyIle-Gly-Asn  
4 -Leu-Ser) and represents a hybrid of the first eight  
5 amino acids from the predicted B cell epitope and the T  
6 cell epitope. The purity of each peptide was confirmed  
7 by C-8 reverse phase HPLC, and all peptides were  
8 desalted over a Sephadex G-10 Column before use. Using  
9 a standard ELISA method, all peptides were assayed for  
10 their ability to specifically bind anti-AF/R1 IgG  
11 antibody in hyperimmune serum from a rabbit which had  
12 received intramuscular injections of AF/R1 pili in  
13 Freund adjuvant. Only the peptide chosen as a probable  
14 B cell epitope (AF/R1 40-55) was recognized by the  
15 hyperimmune serum.

16 EXAMPLE 1

17 Immunization. Rabbits were primed twice with 50  
18 micrograms of either microencapsulated or  
19 non-encapsulated AF/R1 by endoscopic intraduodenal  
20 inoculation seven days apart by the following  
21 technique. All animals were fasted overnight and  
22 sedated with an intramuscular injection of xylazine (10  
23 mg) and Ketamine HCl (50 mg). An Olympus BF type P10  
24 endoscope was advanced under direct visualization  
25 through the esophagus, stomach, and pylorus, and a 2 mm  
26 ERCP catheter was inserted through the biopsy channel



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1 and threaded 2-3 cm into the small intestine.  
2 Inoculums of pili or pili embedded in microspheres were  
3 injected through the catheter into the duodenum and the  
4 endoscope was withdrawn. Animals were monitored daily  
5 for signs of clinical illness, weight gain, or  
6 colonization by RDEC-1.

7 EXAMPLE 2

8 Lymphocyte Proliferation. Seven days following  
9 the second priming, the rabbits were again sedated with  
10 a mixture of xylazine and katamine HCl, and blood was  
11 drawn for serum preparation by cardiac puncture.  
12 Animals were then euthanized with an overdose of  
13 pentothal and tissues including Peyer's patches from  
14 the small bowel, MLN, and spleen were removed. Single  
15 cell suspension were prepared and washed in Dulbeco's  
16 modified Eagle medium (Gibco Laboratories, Grand  
17 Island, NY) which had been supplemented with penicillin  
18 (100 units/ml), streptomycin (100 micrograms/ml),  
19 L-glutamine (2mM), and HEPES Buffer (10 mM) all  
20 obtained from Gibco Laboratories, as well as MEM  
21 non-essential amino acid solution (0.1 mM), MEM [50x]  
22 amino acids (2%), sodium bicarbonate (0.06%), and  $5 \times 10^{-5}$   
23 micrograms 2-ME all obtained from Sigma Chemical  
24 Company (St. Louis, MO) [cDMEM]. Erythrocytes in the  
25 spleen cell suspension were lysed using standard  
26 procedures in an ammonium chloride lysing buffer. Cell

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1 suspension were adjusted to  $5 \times 10^6$  cells per ml in  
2 cDMEM, and autologous serum was added to yield a final  
3 concentration of 0.5%. Cells (0.1 ml) were placed in  
4 96-well flat bottom culture plates (Costar, Cambridge,  
5 MA) along with 0.1 ml of various dilutions of antigen  
6 and were incubated at 37°C in 5% CO<sub>2</sub>. In other  
7 experiments, cultures were conducted in a 24-well  
8 plates. In these experiments,  $5 \times 10^6$  cells were  
9 cultured with or without antigen in a 2 ml volume.  
10 After 4 days, 100 microliters aliquots of cells were  
11 transferred to 96-well plates for pulsing and  
12 harvesting. Previous experiments have demonstrated that  
13 optimal concentrations of antigen range from 150 ng/ml  
14 to 15 micrograms/ml in the 96-well plate assay and 1.5  
15 ng/ml to 150 ng/ml in the 24-well plate assay. These  
16 were the concentrations employed in the current study.  
17 All cultures were pulsed with 1 Ci [<sup>3</sup>H]thymidine (25  
18 Ci/mmol, Amersham, Arlington Heights, IL) on day 4 of  
19 culture and were harvested for scintillation counting 6  
20 hours later.

21 Statistics. All cultures were conducted in  
22 replicates of four, and standard deviations of the  
23 counts per minute (cpm) generally range from 5-15% of  
24 the average cpm. In experiments where comparison of  
25 individual animals and groups of animals is desirable,  
26 data is shown as a stimulation index (SI) to facilitate

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1 the comparison. SI were calculated by dividing the mean  
2 of cultures with antigen by the mean of cultures  
3 without antigen (media control). Statistical  
4 significance (p value) was determined by comparing the  
5 maximum response for each antigen to the media control  
6 using the Student's t test.

7 IX. RESULTS

8 Lymphocyte proliferation in response to protein  
9 and peptide antigens of AF/R1. To determine if  
10 lymphoid tissues from AF/R1 immune animals respond in  
11 vitro to the antigens of AF/R1, the immunity in a  
12 rabbit with preexisting high levels of anti-AF/R1 serum  
13 IgG was boosted twice by injection of 50 micrograms of  
14 purified AF/R1 pili i.p. seven days apart. A week  
15 after the final boost, in vitro lymphocyte  
16 proliferation of spleen and MLN cells demonstrated a  
17 remarkable response to AF/R1 pili (Fig. 13). In  
18 response to the synthetic peptides, there was a small,  
19 but significant proliferation of the spleen cells to  
20 all the AF/R1 peptides tested as compared to cell  
21 cultures without antigen (Fig. 14). Cells from the  
22 spleen and Peyer's patches of non-immune animals failed  
23 to respond to either AF/R1 or the synthetic peptides.

24 Microencapsulation of AF/R1 potentiates the  
25 mucosal cellular immune response. To evaluate the  
26 effect that microencapsulation of AF/R1 may have on the

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1 cellular mucosal immune response to that antigen, naive  
2 rabbits were primed twice with 50 micrograms of either  
3 microencapsulated or non-encapsulated AF/R1 by  
4 endoscopic intraduodenal inoculation seven days apart.  
5 All rabbits were monitored daily and showed no evidence  
6 of clinical illness or colonization by RDEC-1. One  
7 week following the last priming, the rabbits were  
8 sacrificed and lymphoid tissues were cultured in the  
9 presence of AF/R1 pili or peptide antigens. In rabbits  
10 which had received non-encapsulated AF/R1, Peyer's  
11 Patch cells demonstrated a low level but significant  
12 proliferation in vitro in response to AF/R1 pili (Fig.  
13 5), but not to any of the AF/R1 synthetic peptides  
14 (Fig. 6a-6d). However, in rabbits which had received  
15 microencapsulated AF/R1, Peyer's Patch cells  
16 demonstrated a markedly enhanced response not only to  
17 AF/R1 (Fig. 5), but now responded to the AF/R1  
18 synthetic peptides 40-55 and 79-94 (Fig. 6a and 6b).  
19 In addition, one of two rabbits primed with  
20 microencapsulated AF/R1 (rabbit 135) responded to AF/R1  
21 108-123, but not AF/R1 40-47/79-86 (Fig. 6c and 6d).  
22 In contrast, the other rabbit in the group (rabbit 134)  
23 responded to AF/R1 40-47/79-86, but not to AF/R1  
24 108-123 (Fig. 6d and 6c).

25 Response of MLN cells to the antigens of AF/R1.  
26 Studies have shown that cells undergoing blastogenesis

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1 in the MLN also tend to home into mucosal areas, but  
2 experiments requiring in vitro lymphocyte proliferation  
3 of rabbit MLN cells are difficult to conduct and to  
4 interpret due to non-specific high background cpm in  
5 the media controls. Our studies have shown that this  
6 problem can be avoided by conducting the proliferative  
7 studies in 24-well plates, and then moving aliquots of  
8 cells into 96-well plates for pulsing with  
9 [<sup>3</sup>H]thymidine as described in materials and methods.  
10 This method of culture was employed for the remainder  
11 of the studies. The MLN cells of all rabbits  
12 demonstrated a significant proliferation in vitro in  
13 response to AF/R1 pili regardless of whether they had  
14 been immunized with microencapsulated or  
15 non-encapsulated AF/R1 (Fig. 15). However, only the  
16 rabbits which had received microencapsulated AF/R1 were  
17 able to respond to the AF/R1 synthetic peptide 40-55  
18 (Fig. 11). The MLN cells of rabbit 134 also responded  
19 to AF/R1 79-94 ( $p < 0.0001$ ), AF/R1 108-123 ( $p < 0.0001$ ),  
20 and AF/R1 40-47/79-86 ( $p = 0.0004$ ); however, none of the  
21 other rabbits demonstrated a MLN response to those  
22 three peptides (data not shown).

23 Response of spleen cells to the antigens of  
24 AF/R1. Proliferative responses of spleen cells to  
25 AF/R1 were very weak in all animals tested (data not  
26 shown). However, in results which paralleled the

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1        responses in MLN cells, there was a significant  
2        response to AF/R1 40-55 in rabbits which had been  
3        primed with microencapsulated AF/R1 (Fig. 12). There  
4        was no response to the other AF/R1 synthetic peptides  
5        by spleen cells in either group of animals. The weak  
6        response of spleen cells to AF/R1 provides further  
7        evidence that these animals were naive to AF/R1 before  
8        the study began, and indicates that the observed  
9        responses were not due to non-specific stimulative  
10       factors such as lipopolysaccharide.

#### 11                    XI. SUMMARY

12                We have shown that there is an enhanced in vitro  
13        proliferative response to both protein and its peptide  
14        antigens by rabbit Peyer's patch cells following  
15        intraduodenal inoculation of antigen which had been  
16        homogeneously dispersed into the polymeric matrix of  
17        biodegradable, biocompatible microspheres. The  
18        immunopotentiating effect of encapsulating purified  
19        AF/R1 pili as a mucosal delivery system may be  
20        explained by one or more of the following mechanisms:  
21        (a) Microencapsulation may help to protect the antigen  
22        from degradation by digestive enzymes in the intestinal  
23        lumen. (b) Microencapsulation has been found to  
24        effectively enhance the delivery of a high  
25        concentration of antigen specifically into the Peyer's  
26        patch. (c) Once inside the Peyer's patch,

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1 microencapsulation appears to facilitate the rapid  
2 phagocytosis of the antigen by macrophages, and the  
3 microspheres which are 5-10 micrometers become  
4 localized within the Peyer's patch. (d)  
5 Microencapsulation of the antigen may improve the  
6 efficiency of antigen presentation by decreasing the  
7 amount of enzymatic degradation that takes place inside  
8 the macrophage before the epitopes are protected by  
9 combining with Class II major histocompatibility  
10 complex (MHC) molecules. (e) The slow,  
11 controlled-release of antigen may produce a depot  
12 effect that mimics the retention of antigen by the  
13 follicular dendritic cell. (f) If the antigen of  
14 interest is soluble, microencapsulation changes the  
15 antigen into a particulate form which appears to assist  
16 in producing an IgA B cell response by shifting the  
17 cellular immune response towards the  $T_H$  and thereby not  
18 encouraging a response by the  $T_s$ . There is evidence  
19 that the GALT may be able to discriminate between  
20 microbial and non-microbial (food) antigens in part by  
21 the form of the antigen when it is first encountered,  
22 and thus bacterial antigens do not necessarily have  
23 special antigenic characteristics that make them  
24 different from food antigens, but they are antigenic  
25 because of the bacterial context in which they are  
26 presented. The particulate nature of microspheres may

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1       serve to mimic that context. It may be important to  
2       note that we also observed a significant response to  
3       AF/R1 in animals inoculated with non-encapsulated pili;  
4       thus, some of this antigen which was still in its  
5       native form was able to enter the Peyer's patch. This  
6       may be explained by the fact that AF/R1 is known to  
7       mediate the attachment of RDEC-1 to the Peyer's patch  
8       M-cell. If the antigen employed in this type of study  
9       was not able to attach to micrometer M-cells, one would  
10      expect to see an even greater difference in the  
11      responses of animals which had received  
12      microencapsulated versus non-encapsulated antigen.

13           The microspheres used in these experiments  
14      included a size range from 1 to 12 micrometers. The 1  
15      to 5 micrometer particles have been shown to  
16      disseminate to the MLN and spleen within migrating  
17      macrophages; thus, the observed proliferative responses  
18      by cells from the MLN and spleen may reflect priming of  
19      MLN or splenic lymphocytes by  
20      antigen-presenting/accessory cells which have  
21      phagocytosed 1 to 5 micrometer antigen-laden  
22      microspheres in the Peyer's patch and then disseminated  
23      onto the MLN. Alternatively, these responses may be a  
24      result of the normal migration of antigen stimulated  
25      lymphocytes that occurs from the Peyer's patch to the  
26      MLN and on into the general circulation before homing



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1 to mucosal sites. Proliferative responses by MLN cells  
2 are of interest because it has been shown that cells  
3 undergoing blastogenesis in the MLN tend to migrate  
4 onto mucosal areas. However, studies involving in  
5 vitro lymphocyte proliferation of rabbit MLN cells can  
6 be very difficult to conduct and to interpret due to  
7 non-specific high background cpm in the media controls.  
8 By simultaneously conducting experiments using  
9 different protocols, we have found that this problem  
10 can be prevented by avoiding the use of fetal calf  
11 serum in the culture and by initially plating the cells  
12 in 24-well plates. Using this method, the blasting  
13 lymphocytes are easily transferred to a 96-well plate  
14 where they receive the [<sup>3</sup>H]thymidine, while fibroblasts  
15 and other adherent cells remain behind and thus do not  
16 inflate the background cpm.

17 The proliferative response to the peptide  
18 antigens was of particular interest in these studies.  
19 The rabbits that received non-encapsulated AF/R1 failed  
20 to respond to any of the peptides tested either at the  
21 level of the Peyer's patch, the MLN, or the spleen. In  
22 contrast, Peyer's patch cells from the animals that  
23 received microencapsulated AF/R1 responded to all the  
24 peptides tested with two exceptions: Rabbit 134 did  
25 not respond to AF/R1 108-123, and rabbit 135 did not  
26 respond to AF/R1 40-47/79-86. The reason for these

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1 non-responses is not clear, but it probably is not due  
2 to MHC restrictions as evidenced by the fact that  
3 rabbit 134 was able to respond to AF/R1 108-123 at the  
4 level of the MLN. The non-responses may be due to  
5 varying kinetics of sensitized T cell migration in  
6 different rabbits, or they may reflect differences in  
7 the efficiency of antigen presentation by cells from  
8 different lymphoid tissues of these animals. Of all  
9 the synthetic peptides tested, only AF/R1 40-55, (the  
10 one selected as a probable B cell epitope), was  
11 recognized by serum from an AF/R1 hyperimmune rabbit.  
12 In addition, this peptide was the only one that was  
13 uniformly recognized by Peyer's patch, MLN, and spleen  
14 cells from both rabbit. In addition, this peptide was  
15 the only one that was uniformly recognized by Peyer's  
16 patch, MLN, and spleen cells from both rabbits that  
17 were immunized with microencapsulated AF/R1. The  
18 recognition by anti-AF/R1 serum antibodies indicates  
19 that the amino acid sequence of this peptide includes  
20 an immunodominant B cell epitope. Thus AF/R1 40-55 may  
21 readily bind to antigen-specific B cells thereby  
22 leading to an efficient B cell presentation of this  
23 antigen to sensitized T cells. Even though AF/R1 40-55  
24 was not selected as a probable T cell epitope by either  
25 the Rothbard or Berzofsky methods, the current study  
26 clearly indicates that this peptide can also stimulate

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1 a proliferative immune response. Although further  
2 studies are required to definitively show that the  
3 proliferating cells are indeed T cells, the responses  
4 observed in this study are most likely due to the blast  
5 transformation of cells from the lineage. Therefore,  
6 AF/R1 40-55 appears to contain a T cell epitope in  
7 addition to the immunodominant B cell epitope, and this  
8 area of the AF/R1 protein may thereby play an important  
9 role in the overall immune response and subsequent  
10 protection against RDEC-1.

11 The proliferative responses of spleen cells was  
12 low in all animals tested; however, we feel tht this  
13 may be simply a matter of the kinetics of cellular  
14 migration. The rabbits in this study were sacrificed  
15 only two weeks after their first exposure to antigen.  
16 This relatively short time period may not have provided  
17 sufficient time for cells that were produced by Peyer's  
18 patch and MLN blasts to have migrated as far as the  
19 spleen in sufficient numbers.

20 An ideal mucosal vaccine preparation would not  
21 only assist in the uptake and presentation of the  
22 immunogen of interst, but it would also be effective  
23 without requiring carrier molecules or adjuvants which  
24 may complicate vaccine production or delay regulatory  
25 approval. The incorporation of antigen into  
26 microspheres appears to provide an ideal mucosal

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1 delivery system for oral vaccine immunogens because the  
2 observed immunopotentiating effect is achieved without  
3 the need for carriers of adjuvants. This ability may  
4 prove to be of great value, particularly to enhance the  
5 delivery of oral synthetic peptide vaccines to the  
6 GALT.

7 TABLE 1. Linear B-Cell Epitopes of CFA/I in Monkeys

8	Sequence	Individuals	
9	<u>Position</u>	<u>Responding</u>	<u>Consensus Site</u>
10	1. 11-21	3	VDPVIDLLQ
11	2. 93-101	2	AKEFEAAA
12	3. 124-136	2	GPAPT
13	4. 66-74	2	PQLTDVLN
14	5. 22-29	2	GNALPSAV
15	6. 32-40	1	KTF*
16	7. 38-45	1	
17	8. 3-11	1	

18

19

---

\*Overlap between epitope 6 and 7

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TABLE 2

1			
2		<u>Prediction of T cell epitopes within the CFA/I</u>	
3		<u>molecule'</u>	
4		<u>Predicted Amphipathic Segments</u>	<u>Rothbard Criteria</u>
5		7 aa blocks	11 aa blocks
6			
7		22-25	8-11
8	16		
9		34-38	32-44
10	30		
11		40-46	51-71
12	38		
13		50-53	86-92
14	44		
15		56-62	102-108
16	57		
17		64-71	130-131
18	61		
19		104-108	135-137
20	70		
21		131-137	
22	116		
23			124
24			127
25			137

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1           \*The sequence numbers of the first amino acid of  
 2           the predicted T cell epitopes are shown. Software  
 3           designed to predict T cell epitopes based on the  
 4           Berzofsky method was published as the AMPHI program.  
 5           It predicts amphipathic amino acid segments by  
 6           evaluating 7 or 11 residues as a block and assigning a  
 7           score to the middle residue of that block. Software  
 8           designed to predict T cell epitopes based on the  
 9           Rothbard method was written by Stephen Van Albert (The  
 10          Walter Reed Army Institute of Research, Washington,  
 11          D.C.).

TABLE 3

13          Amino acid sequence of immunodominant T cell epitopes\*

14	Residue	
16	Numbers	Amino Acids
17		
18	8-17	Thr Ala Ser Val Asp Pro Val Ile Asp Leu
19	40-49	Phe Glu Ser Tyr Arg Val Met Thr Gln Val
20	72-81	Leu Asn Ser Thr Val Gln Met Pro Ile Ser
21	134-143	Asn Tyr Ser Gly Val Val Ser Leu Val Met

23           \*Of the 19 decapeptides that supported a  
 24           significant proliferative response and contained a  
 25           serine at either position 2, 3, or 4, nine has a serine  
 26           specifically at position 3. Some of the most robust

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1 responses were to the peptides that contained a serine  
2 residue at the third position. The amino acid sequence  
3 of four such decapeptides which are believed to be  
4 immunodominant T cell epitopes is shown.

5 PHASE III

6 The development of a safe and effective vaccine  
7 against enterotoxigenic E. coli (ETEC) would be useful  
8 for travelers and for young children in endemic areas.  
9 A phase I study of an enteral ETEC vaccine candidate  
10 consisting of colonization factor antigen II (CFA/II)  
11 encapsulated in biodegradable polymer microspheres  
12 (BPM) was conducted in healthy volunteers.

13 Ten adult volunteers swallowed intestinal tubes  
14 on days 0, 7, 14, and 28; after collection of jejunal  
15 fluid samples, 1 mg of CFA/II in BPM was administered  
16 via the tube. Volunteers kept a diary of symptoms  
17 after each dose. Secretory IgA in jejunal fluids,  
18 serum responses, and antibody secreting cells (ASC)  
19 were measured before and after vaccination.

20 The vaccine was well tolerated. Five of 10  
21 volunteers had developed IgA anti-CFA/II ASC by 7 days  
22 after the last dose of vaccine, these same 5 vaccinees  
23 had IgA anti-C63 ASC, and 3 of 5 vaccinees had IgA  
24 anti-CS1 ASC. Five of 10 vaccinees developed rises in  
25 jejunal fluid sIgA anti-CFA/II with peak CMT of 1:42.  
26 Serum responses were meager. Ten vaccinees and 10

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1       unvaccinated control volunteers underwent challenge  
2       with  $10^9$  cfu ETEC E24377A (0139;H2B LT+ST+CS1+CS3+).  
3       Ten of 10 controls and 7 of 10 vaccinees developed  
4       diarrhea ( $p=.11$ , 30% vaccine efficacy). One of the 3  
5       protected vaccinees had the highest number of ASC and  
6       highest sIgA titer before challenge, suggesting that  
7       these responses were protective and that this vaccine  
8       development strategy has merit. Future studies with  
9       higher dosages and a different dosing schedule are  
10      planned.

11             Enterotoxigenic Escherichia coli (ETEC) is  
12      responsible for diarrhea in infants in developing  
13      countries and for a large proportion of diarrhea among  
14      travelers to developing countries. Development of a  
15      vaccine against ETEC is therefore an important public  
16      health priority. Studies in animals and challenged  
17      volunteers suggest that orally administered fimbriae,  
18      which function as colonization factors, should induce  
19      protective immunity.

20             An ETEC vaccine candidate was developed which  
21      consists of purified colonization factor antigen II  
22      (CFA/II) derived from ETEC strain M424 (06:H16:K15)  
23      encapsulated in biodegradable polymer microspheres  
24      (BPM). CFA/II from this strain consists of two surface  
25      structures, a fibrillar designated coli surface antigen  
26      1 (CS1) and a fibrillar structure designated coli



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1 surface antigen 3 (CS3). The purpose of encapsulating  
2 the antigen into microspheres is to protect it during  
3 passage through the stomach and to enhance its uptake  
4 by gut-associated lymphoid tissues (GALT), such as  
5 Peyer's patches. The microspheres consist of a 50:50  
6 copolymer of lactic and glycolic acids (DL-lactide-co-  
7 glycolide). In animals, antigens delivered in these  
8 microspheres are taken up and processed by the GALT and  
9 stimulate vigorous local immune responses.

10 In this report we describe the safety,  
11 Immunogenicity, and efficacy against experimental  
12 challenge of the CFA/II-BPM vaccine in healthy  
13 volunteers. This is the first use in man of this  
14 delivery system for an oral antigen.

15 This phase III describes the result of E. coli  
16 CVD 15000, a clinical study of the safety,  
17 immunogenicity, and efficacy against experimental  
18 challenge of a new vaccine against enterotoxigenic E.  
19 coli (ETEC). This vaccine consists of colonization  
20 factor antigen II (CFA/II) purified from ETEC strain  
21 M424 (06:H16:K15) encapsulated in biodegradable polymer  
22 microspheres (CFA/II-BPM).

### 23 MATERIALS AND METHODS

24 CFA/II-BPM vaccine was prepared at the University  
25 of Maryland School of Pharmacy. Each dose of vaccine  
26 consisted of 1 mg of CFA/II (90% CS3, 10% CS1)

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1 incorporated into 100 mg of BPM 1. 10 microns in  
2 diameter; the freeze-dried microspheres were dispersed  
3 in saline containing 0.5% polysorbate<sub>60</sub>. Ten healthy  
4 adult outpatient volunteers were recruited for  
5 vaccination with four doses of CFA/II-BPM vaccine.  
6 Each volunteer swallowed an intestinal tube on days 0,  
7 7, 14, and 28; after collection of jejunal fluid,  
8 CFA/II-BPM was administered via the tube. The vaccine  
9 vials were sonicated immediately before vaccination to  
10 achieve an even suspension of the turbid vaccine.

11 Volunteers kept a diary of symptoms for five days  
12 after each dose of vaccine. Jejunal fluids were  
13 collected via intestinal tube on days 0, 7, 14, 28, and  
14 35 after vaccination for measurement of secretory IgA.  
15 Whole blood for antibody secreting cells (ASC) was  
16 collected on days 0, 7, 14, 21, and 35. Serum was  
17 collected for antibody against CFA/II on days 0, 7, and  
18 28. ASC responses were measured by ELISPOT assays  
19 using a variety of antigens: CFA/II vaccine antigen  
20 derived from ETEC strain M424 (06:H16:K15 CS1+CS3+),  
21 purified CS1 derived from ETEC strain 60R75 (O:H CS1+),  
22 and purified CS3 derived from ETEC strain E9034 (08:H9  
23 CS3+). Four or more spots was considered a significant  
24 number. Serum antibody measurements to CFA/II,  
25 purified CS1, and purified CS3 were performed by ELISA.  
26 A four-fold rise in titer was considered significant.

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1        Jejunal fluids were adjusted to a concentration of IgA  
2        of 20 mg% and then lyophilized before assaying for  
3        specific anti-CFA/II activity.

4                Fifty-seven days after the first dose of CFA/II-  
5        BPM vaccine, 10 vaccinees and 10 unimmunized control  
6        volunteers were admitted to the Research isolation Ward  
7        in the University of Maryland Hospital. After  
8        screening for excellent health, volunteers ingested 3 x  
9        10<sup>9</sup> cfu of ETEC strain E24377A (O139:H28  
10       LT+ST+CS1+CS3+) with sodium bicarbonate. Blood samples  
11       were collected for serologic responses to CFA/II,  
12       O139(LPS) antigen, and heat labile enterotoxin (LT)  
13       before and on days 14 and 28 after challenge. Jejunal  
14       fluids for measurement of sigA against CFA/II were  
15       collected before and on day 7 after challenge.

16                Part I: Outpatient Vaccination Study

17                Ten healthy adult outpatients volunteers were  
18        recruited for vaccination with CFA/II-BPM vaccine.  
19        Each volunteer swallowed an intestinal tube on  
20        September 2, 9, 16, and 30 (days 0, 7, 14, and 28);  
21        after collection of jejunal fluid, 1 mg of CFA/II in  
22        BPM was administered via the tube. The vaccine was  
23        prepared immediately before vaccination as directed by  
24        Dr. Reid; specifically, the vials were sonicated to  
25        achieve an even suspension of the turbid vaccine. For  
26        two volunteers, one or more doses of vaccine had to be

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1 administered intragastrically (noted in data tables)  
2 because the tube failed to move out of the stomach  
3 after over 56 hours of intubation.

4 **Safety.** Volunteers kept a diary of symptoms for  
5 five days after each dose of vaccine. The vaccine was  
6 well tolerated. One volunteer reported mild cramps for  
7 15 minutes on day 1 after the second dose. A second  
8 volunteer reported cramps lasting for about one hour  
9 before passing loose stools on days 3 and 4 after the  
10 second dose; the volunteer attributed this to having  
11 eaten crabs.

12 **Immunogenicity.** Jejunal fluids were collected  
13 via intestinal tube on days 0, 8, 14, 28, and 35 after  
14 vaccination for measurement of secretory IgA. Whole  
15 blood for antibody secreting cells (ASC) was collected  
16 on days 0, 7, 14, 21, and 35. Serum was collected for  
17 antibody determinations on days 0, 7, and 28. Whole  
18 blood for measuring T cell responses by lymphocyte  
19 transformation were drawn on days 0 and 35 after  
20 vaccination.

21 **ASC.** Detection of CFA/II-specific antibody  
22 secreting cells in peripheral blood reflects priming of  
23 the intestinal mucosal immune system; these cells have  
24 been stimulated by oral antigen, entered the  
25 circulation, and are returning to the mucosa to provide

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1 local immunicyt. The role of these cells in protection  
2 against ETEC diarrhea is unknown.

3 We measured ASC responses by ELISPOT assays  
4 using a variety of antigens: CFA/II vaccine antigen  
5 derived from ETEC strain M424 (06:H16:K15 CS1+CS3+),  
6 purified CD1 derived from ETEC strain 60R75 (0:H CS1+),  
7 purified CS3 derived from ETEC strain E9034 (08:H9  
8 CS3+), CS3 peptide 795, CS3 peptide 792, and as  
9 controls, CFA/I, CFA/I peptide 791, and CFA/I peptide  
10 900. The results of these assays are shown in Tables 1  
11 through 5. Four or more spots is considered a  
12 significant number.

13 At day 7 after the first dose of vaccine, four of  
14 the 10 volunteers developed IgA ASC against CFA/II  
15 (Table 1). After the second and third doses of vaccine  
16 no additional responders were detected. However, after  
17 the fourth dose, an additional volunteer developed a  
18 significant response so that the overall response after  
19 four doses of CFA/II-BPM was five (50%) of 10  
20 vaccinees.

21 Three of the volunteers who responded with IgA  
22 ASC against CFA/II also had IgA ASC against purified  
23 CS1 (Table 8). The same five volunteers who responded  
24 to CFA/II also had IgA ASC against purified CS3 (Table  
25 9). This suggests that the responses to CFA/II were  
26 specific and not directed against contaminating

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elements such as LPS, since the serotypes of the strains from which the antigens were prepared were different. IgA ASC responses to two peptides derived from CS3 were meager or absent (Tables 10 and 11). There were no ASC responses to CFA/I or to two peptides derived from CFA/I. This is further evidence that the responses to CFA/II were not directed against contaminating elements in the antigen preparations.

Jejunal fluid sigA. After the first dose of CFA/II-BPM vaccine, only one volunteer developed a rise in sigA to CFA/II and this volunteer (15001-9) had evidence of previous priming since his pre-vaccination sigA anti-CFA/II titer was 1:16 (Table 12). One week after the fourth dose (day 35), however, five of the 10 vaccinees had developed rises in sigA anti-CFA/II. Among these five converters, the peak geometric mean titer was 1:42.

**Serology.** Serum antibody measurements to CFA/II, purified CS1, and purified CS3 were also performed by ELISA. A four-fold rise in titer was considered significant and indicated by a + in the tables. There was a high prevalence of serum antibody to CFA/II before vaccination (Table 13); only two of 10 volunteers developed rises in serum IgA anti-CFA/II and a third volunteer developed a rise in serum IgG anti-CFA/II. Only one volunteer developed serum antibody to

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1 CS1 (Table 14). However, six of the 10 vaccinees  
2 developed seroconversions to anti-CS3 with antibody of  
3 at least one isotpy (Table 15).

4 **Lymphocyte proliferation studies.** Lymphocytes  
5 were separated from whole blood on ficoll-hypaque  
6 gradients and stored forzen for future proliferative  
7 assays by Dr. Reid at WRAIR.

8 **Part II: Experimental ETEC Challenge Study**

9 All 10 vaccinees and 10 control volunteers agreed  
10 to participate in an ETEC challenge. One October 29,  
11 1992, 57 days after the first dose of CFA/II-BPM  
12 vaccine, 20 volunteers ingested  $3 \times 10^9$  cfu of ETEC  
13 strain E24377A (0139:H28 LT+ST+CS1+CS3+). The clinical  
14 and bacteriologic responses to challenge are shown in  
15 Table 10.

16 Ten of 10 control volunteers and seven of 10  
17 vaccines developed diarrhea ( $p=0.11$ , Fisher's exact  
18 test, 1-tailed; 30% vaccine efficacy). the mean volume  
19 of diarrheal stools was 1464 ml for controls and 2819  
20 ml for vaccines ( $p=0.2$ , Student's t test); the mean  
21 number of diarrheal stools was 8.6 for controls and  
22 14.7 for vaccinees ( $p=0.2$ , Student's t test). The mean  
23 incubation periods in the two groups were not  
24 significantly different. The duration of stool  
25 shedding and the peak stool excretion of challenge  
26 organisms were not significantly different. The three

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1       protected vaccinees had a somewhat lower peak excretion  
2       of challenge organisms than the seven unprotected  
3       vaccinees, but this difference was not statistically  
4       significant.

5               Before challenge (day 57 after the first dose of  
6       vaccine), the three protected vaccinees, five vaccinees  
7       who became ill, and four control volunteers who became  
8       ill had circulating ASC producing antibodies of some  
9       isotype against CFA/II, CS1, or CS3 (Table 17). The  
10       vaccinee (volunteer 15001-9) with the highest number of  
11       IgA anti-CFA/II ASC (240 spots) before challenge and  
12       the highest number of IgA anti-CS3 ASC (16 spots)  
13       before challenge was one of the three protected  
14       vaccinees. The other protected vaccinees (volunteers  
15       15001-6 and 15001-11) had no detectable anti-CFA/II IgA  
16       ASC before challenge but did have anti-CS1 ASC or anti-  
17       CS3 IgA ASC. Conversely, unvaccinated control  
18       volunteers with pre-existing IgA anti-CFA/II ASC were  
19       not protected (e.g., volunteers 15002-8, 15002-11, and  
20       15002-13).

21               The level of ASC response inducted by infection  
22       provides a target for future vaccine-induced immunity.  
23       After wild-type challenge of vaccinees and controls,  
24       IgA ASC responses to CFA/II and CS3 were vigorous  
25       (range 12-408 spots for CFA/II and 14-712 spots for  
26       CS3) (Table 17). After challenge one vaccinee and one



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1 control volunteer mounted ASC responses to CS3 peptide  
2 792 (Table 18). Four vaccinees (15001-1, 15001-6,  
3 15001-7, and 15001-11) and one control volunteer  
4 (15002-11) developed a small number of ASC to CS3  
5 peptide 795 (Table 18).

6 There was no correlation between pre-existing  
7 anti-LPS ASC and protection (Table 19). None of the  
8 three protected vaccinees had such antibodies before  
9 challenge. Two volunteers with pre-existing anti-LPS  
10 ASC nevertheless became ill (volunteers 15001-1 and  
11 15001-8). Similarly, there was not correlation between  
12 protection against illness and pre-existing anti-LT ASC  
13 (Table 19).

14 The serologic responses and jejunal fluid  
15 antibody responses to challenge are pending at the time  
16 of this writing. These results will be summarized in  
17 an addendum to this report.

## 18 RESULTS

19 Clinical and immunologic responses to  
20 vaccination. The vaccine was well tolerated. For two  
21 volunteers, four doses of vaccine had to be  
22 administered intragastrically in two volunteers because  
23 the tube failed to move out of the stomach after over  
24 56 hours of intubation.

25 Detection of CFA/II-specific antibody secreting  
26 cells in peripheral blood reflects priming of the

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1       intestinal mucosal immune system; these cells have been  
2       stimulated by oral antigen, entered the circulation,  
3       and are refurring to the mucosa to provide local  
4       immunity. At day 7 after the first dose of vaccine,  
5       four of the 10 volunteers developed IgA ASC against  
6       CFA/II. Ater the second and third doses of vaccine no  
7       additional responders were detected. However, after  
8       the fourth dose, an additional volunteer developed a  
9       significant response so that the overall response after  
10      four doses of CFA/II-BPM was five (50%) of 10 vaccinees  
11      by day 35 (Table 20). Three of the volunteers who  
12      responded with IgA ASC against CFA/II also had IgA ASC  
13      against purified CS1 (Table 20). The same five  
14      volunteers who responded to CFA/II also had IgA ASC  
15      against purified CS3 (Table 20). This suggests that  
16      the responses to CFA/II were specific and not directed  
17      against contaminating elements such as LPS, since the  
18      serotypes of the strains from which the antigens were  
19      prepared were different.

20           After the first dose of CFA/II-BPM vaccine, only  
21      one volunteer developed a rise in jejunal fluid sigA to  
22      CFA/II, and this volunteer had evidence of previous  
23      priming since his pre-vaccination sigA anti-CFA/II  
24      titer was 1:16. One week after the fourth dose (day  
25      35), however, five of the 10 vaccinees had developed

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1 rises in sigA anti-CFA/II (Table 20). Among these five  
2 converters, the peak geometric mean titer was 1:42.

3 There was a high prevalence of serum antibody to  
4 CFA/II before vaccination; only two of 10 volunteers  
5 developed rises in serum IgA anti-CFA/II and a third  
6 volunteer developed a rise in serum IgG anti-CFA/II.  
7 Only one volunteer developed serum antibody to CS1.  
8 However, six of the 10 vaccinees developed  
9 seroconversions to anti-CS3 with antibody of at least  
10 one isotype.

11 **Clinical and bacteriologic responses to**  
12 **experimental ETEC challenge.** Fifty-seven days after  
13 the first dose of CFA/II-BPM vaccine, 10 vaccinees and  
14 10 control volunteers ingested  $3 \times 10^9$  cfu of ETEC  
15 strain E24377A (0139:H28 LT<sup>+</sup>ST<sup>+</sup>CS1<sup>+</sup>CS3<sup>+</sup>). The  
16 immunologic status at the time of challenge and the  
17 clinical and bacteriologic responses to challenge are  
18 shown in Table 22.

19 Ten of 10 control volunteers and seven of 10  
20 vaccinees developed diarrhea ( $p=0.11$ , Fisher's exact  
21 test, 1-tailed; 30% vaccine efficacy). The mean volume  
22 of diarrheal stools was 1464 ml for controls and 2819  
23 ml for vaccines ( $p=0.2$ , Student's t test); the mean  
24 number of diarrheal stools was 8.6 for controls and  
25 14.7 for vaccinees ( $p=0.2$ , Student's t test). The mean  
26 incubation periods in the two groups were not

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1 significantly different. The duration of stool  
2 shedding and the peak stool excretion of challenge  
3 organisms were not significantly different.

4 On the day of challenge, 8 of 10 vaccinees and 4  
5 of the 10 control volunteers had circulating IgA ASC  
6 producing antibodies against CFA/II, CS1, and/or CD3.  
7 The apparent development of additional ASC responders  
8 on day 57 after the first dose of vaccine (making the  
9 total number of vaccine responders 8 of 10) was  
10 unexpected. The high prevalence of anti-colonization  
11 factor ASC in control volunteers before challenge was  
12 also unexpected and not observed in previous groups of  
13 North American volunteers. The vaccinees with the  
14 highest number of IgA anti-CFA/II ASC (240 spots), the  
15 highest number of IgA anti-CS3 ASC (16 spots), and the  
16 highest sigA anti-CFA/II titer (1:256) before challenge  
17 was one of the three protected vaccinees. Conversely,  
18 the 4 unvaccinated control volunteers with pre-existing  
19 IgA anti-CFA/II ASC (range 8-32 spots) were not  
20 protected; none of these had pre-existing sigA measured  
21 in jejunal fluid before challenge.

22 There was no correlation between pre-existing  
23 anti-LPS ASC and protection. Similarly, there was no  
24 correlation between protection against illness and pre-  
25 existing anti-LT ASC.

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1           Immune responses after wild-type challenge, which  
2           are likely to be protective against subsequent  
3           challenge, are a target for vaccine-induced immunity.  
4           The immune responses in volunteers after 4 doses of  
5           CFA/II-BPM vaccine (Table 20) can be compared to those  
6           of unimmunized control volunteers after challenge  
7           (Table 21). Responses after this vaccine regimen  
8           occurred at a lower rate and were of lower magnitude  
9           than those achieved after a vigorous wild-type  
10          challenge.

#### 11           DISCUSSION

12          CFA/II-BPM vaccine was well tolerated in adult  
13          volunteers. When immune responses were measured by the  
14          presence of IgA ASC or jejunal fluid sigA, both  
15          measured 7 days after the fourth dose, half the  
16          volunteers responded to four doses of 1 mg CFA/II-BPM  
17          per dose. The vaccine conferred 30% protective  
18          efficacy against a rigorous experimental challenge that  
19          produced an attach rate of 100% in control volunteers.

20          The three protected vaccinees did not differ  
21          significantly from the seven unprotected vaccinees, at  
22          least in the immune parameters measured in this study.  
23          However, the volunteer who had the highest number of  
24          ASC against CFA/II and CS3 and the highest sigA titers  
25          among the vaccinees was one of the 3 vaccinees who did

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1 not become ill. This suggests that these immune  
2 responses contributed to protection.

3 Some volunteers had a significant number of IgA,  
4 IgG, or IgM ASC to CFA/II and/or CS3 on day 57 after  
5 the first dose of vaccine (the day of challenge) that  
6 were not present on day 35 after vaccination. This  
7 suggests that the biodegradable polymer microspheres  
8 may have persisted in the submucosa and continued to  
9 stimulate responses beyond the 7 to 10 days when ASC  
10 responses are ordinarily expected. However, some  
11 control volunteers also had ASC responses to CFA/II  
12 before challenge. No technical difficulty with the ASC  
13 assay could be identified and control blank wells did  
14 not react. Confirmation of the presistence of CFA/II-  
15 BPM vaccine with continued induction of immune  
16 responses will await future studies.

17 The modest efficacy of CFA/II-BPM vaccine may be  
18 related to the very small doseage (1 mg of Cfa/II x 4  
19 doses) given. The responses after ETEC challenge  
20 summarized in Table 21. However, are within reach,  
21 perhaps by increasing the dose or changing the schedule  
22 of vaccination. Future studies should also include  
23 evaluation of the oral route of administration because  
24 of the impracticality of delivering vaccine via  
25 intestinal tube.

26 DEMONSTRATIVE EVIDENCE OF PROTECTIVE IMMUNITY

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1 RDEC-1 is an eteroadherent diarrhea producing E.  
2 coli in rabbit. Its attachment to the mucosa is by the  
3 adhesin (AF/R1 pili). The adhesin is an excellent  
4 vaccine candidate. It may intitiate a mucosal response  
5 but is susceptiple to digestion in the gut. The  
6 incorporation of AF/R1 into biocompabile,  
7 nondigestible microspheres enhanced mucosal cellular  
8 immune respones to RDEC-1. We have demonstrated that  
9 immunization with AF/R1 Pili in microspheres protect  
10 rabbits against infection with RDEC-1.

11 Six rabbits received intra-duodenal immunizaiton  
12 of AF/R1 microspheres (0.62% coreloading by weight) at  
13 200 ug AF/R1 on day 0 then boosted with 100 ug AF/R1 in  
14 microspheres on days 7, 14, and 21 followed by RDEC-1  
15 challenge with  $10^8$  organisms one week latter than  
16 observed for 1 week and then sacrificed, unimmunized  
17 rabbits were challenged with  $10^8$  RDEC-1 only and  
18 observed 1 week than sacrificed. Also, 2 rabbits were  
19 immunized only then were sacrificed 10 days latter.  
20 Only one of these animals had bile IgA antibodies to  
21 AF/R1. but both had specific sensitized T cells which  
22 released IL-4 upon challenge in the spleen, Peyer's  
23 patch and illeal lamina propria. All nine immunized  
24 animals developed diarrhea and weight loss which was  
25 significant at the  $p < .001$  level compared to the  
26 immunized animals which displayed no diarrhea and no

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1 weight loss. The immunized animals colonized the  
2 intestinal tract with RDEC-1 the same as the  
3 unimmunized animals. However, there was a striking  
4 difference regarding the adherence of RDEC-1 to the  
5 mucosa. No adherence was seen in cecum in the  
6 immunized animals compared to 4/7 in the unimmunized  
7 side animals. This difference was significant to the p  
8 < .01 level. The RDEC-1 exposure although not  
9 producing disease in the immunized animals did effect a  
10 booster immunization as relected in the increase in  
11 anti-AF/R1 antibody containing cells in the muscosa  
12 similiar to the immunized rabbits. This study clearly  
13 demonstrated complete protection against RDEC-1  
14 infection and strongly indicates similiar results  
15 should be expected with enterotoxigenicity E. coli using  
16 the Colony Forming Antigens (CFA's) in microsphere  
17 vaccines.

18 SUMMARY STATEMENT OF PROTECTIVE IMMUNITY SHOWINGS

19 RDEC-1 infection of rabbits causes an  
20 enteroadherent E. coli diarrheal disease, and provides  
21 a model for the study of adherence-factor immunity.  
22 Pilus adhesions are vaccine candidates, but purified  
23 pili are subject to intestinal degradation. Previously  
24 we showed potentiation of the mucosal cellular immune  
25 response to the AF/R1 pilus of RDEC-1 by incorporation  
26 into biodegradable polylactide-coglycolide microspheres



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1 (AF/R1-MS). We now present efficacy testing of this  
2 vaccine. Six rabbits were primed with 200 ug and  
3 boosted with 100 ug of AF/R1-MS weekly x3, then  
4 challenged at week 5 with  $10^8$  CFU of RDEC-1 expressing  
5 AF/R1. Nine unvaccinated rabbits were also challenged.  
6 Two rabbits vaccinated with AF/R1-MS were sacrificed at  
7 week 5, without challenge, for measurement of  
8 anti-AF/R1 antibodies in bile (by ELISA) and anti-AF/R1  
9 containing cells (ACC) in the intestinal lamina propria  
10 (by immunohistochemistry). Attachment of RDEC-1 to  
11 intestinal epithelial cells was estimated (0.4+) by  
12 immunoperoxidase staining of histologic sections.  
13 Colonization of intestinal fluid was measured by  
14 culture of intestinal flushes. Results: Rabbits given  
15 AF/R1-MS remained well and 4/6 gained weight after  
16 challenge, whereas 9/9 unvaccinated rabbits lost weight  
17 after challenge (mean weight change +10 vs -270 gms  
18  $p < .001$ ), (see Figure 27). The mean score of RDEC-1  
19 attachment to the cecal epithelium was 0 in vaccinated,  
20 and 2+ in unvaccinated animals (see Figure 28). RDEC-1  
21 colonization (log CFU/gm) in cecal fluids was similar  
22 in both groups (mean 6.3 vs 7.3;  $p = .09$ ) (see Figure  
23 26). ACC were not seen in the lamina propria of  
24 vaccinated but unchallenged animals, but anti-pilus IgA  
25 antibody levels in bile were increased 1 S.D. over  
26 negative controls in 1 animal. Conclusions:

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1       Vaccination with AF/R1-MS was safe and protected  
2       rabbits against RDEC-1 disease. Protection was  
3       associated with interference with RDEC-1 adherence to  
4       the mucosal surface, but luminal colonization was not  
5       prevented.

6               More recently, applicants have focused on areas  
7       of this invention related to an immunostimulating  
8       composition comprising encapsulating microspheres,  
9       which may contain a pharmaceutically-acceptable  
10       adjuvant, wherein said microspheres are comprised of  
11       (a) a biodegradable-biocompatible poly (DL-lactide-co-  
12       glycolide) as the bulk matrix, wherein the relative  
13       ratio between the amount of lactide and glycolide  
14       components are within the range of 40:60 to 0:100 and  
15       (b) an immunogenic substance comprising Colony Factor  
16       Antigen (DFA/II, hepatitis B surface antigen (HBsAg),  
17       or a physiologically similar antigen that serves to  
18       elicit the production of antibodies in mammalian  
19       subjects.

20               These areas of invention are referred to herein  
21       after as Phase III and Phase IV, respectively, and are  
22       summarized as follows:

23               1. An immunostimulating composition comprising  
24       encapsulating- microspheres, which may contain a  
25       pharmaceutically-acceptable adjuvant, wherein said  
26       microspheres having a diameter between 1 nanogram (ng)

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1 to 10 microns (um) are comprised of (a) a  
2 biodegradable-biocompatible poly (DL-lactide-co-  
3 glycolide) as the bulk matrix, wherein the relative  
4 ratio between the amount of lactide and glycolide  
5 components are within the range of 40:60 to 0:100 and  
6 (b) an immunogenic substance comprising Colony Factor  
7 Antigen (CFA/II), hepatitis B surface antigen (HBsAg),  
8 or a physiologically similar antigen that serves to  
9 elicit the production of antibodies in animal subjects.

10 2. An immunostimulating composition according to  
11 Claim 1 wherein the amount of said immunogenic  
12 substance is within the range of 0.1 to 1.5% based on  
13 the volume of said bulk matrix.

14 3. An immunostimulating composition according to  
15 Claim 2 wherein the relative ratio between the lactide  
16 and glycolide component is within the range of 48:52 to  
17 58:42.

18 4. An immunostimulating composition according to  
19 Claim 2 wherein the size of more than 50% of said  
20 microspheres is between 5 to 10 um in diameter by  
21 volume.

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1           5. An immunostimulating composition according  
2 to Claim 1 wherein the immunogenic substance is the  
3 synthetic peptide representing the peptide fragment  
4 beginning with the amino acid residue 63 through 78 of  
5 Pilus Protein CS3, said residue having the amino acid  
6 sequence, 63(Ser-Lys-Asn-Gly-Thr-Val-Thr-Try-Ala-His-  
7 Glu-Thr-Asn-Asn-Ser-Ala).

8           6. A vaccine comprising an immunostimulating  
9 composition of Claim 4 and a sterile, pharmaceutically-  
10 acceptable carrier therefor.

11           7. A vaccine comprising an immunostimulating  
12 composition of Claim 6 wherein said immunogenic  
13 substance is Colony Factor Antigen (CFA/II).

14           8. A vaccine comprising an immunostimulating  
15 composition of Claim 6 wherein said immunogenic  
16 substance is hepatitis B surface antigen (HBsAg).

17           9. A method for the vaccination against  
18 bacterial infection comprising administering to a  
19 human, an antibactericidally effective amount of a  
20 composition of Claim 6.

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1           10. A method according to Claim 8 wherein the  
2 bacterial infection is caused by a bacteria selected  
3 from the group consisting essentially of Salmonella  
4 typhi, Shigella Sonnei, Shigella Flexneri, Shigella  
5 dysenteriae, Shigella boydii, Escheria coli, Vibrio  
6 cholera, yersinia, staphylococcus, clostridium, and  
7 campylobacter.

8           11. A method for the vaccination against viral  
9 infection comprising administering to a human an  
10 antivirally effective amount of a composition of Claim  
11 8.

12           12. A diagnostic assay for bacterial infections  
13 comprising a composition of Claim 4.

14           13. A method of preparing an immunotherapeutic  
15 agent against infections caused by a bacteria  
16 comprising the step of immunizing a plasma donor with a  
17 vaccine according to Claim 7 such that a hyperimmune  
18 globulin is produced which contains antibodies directed  
19 against the bacteria.

20           14. A method preparing an immunotherapeutic  
21 agent against infections caused by a virus comprising  
22 the step of immunizing a plasma donor with a vaccine

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1           according to Claim 8 such that hyperimmune globulin is  
2           produced which contains antibodies directed against the  
3           hepatitis B virus.

4                   15. An immunotherapy method comprising the step  
5           of administering to a subject an immunostimulatory  
6           amount of hyperimmune globulin prepared according to  
7           Claim 13.

8                   16. An immunotherapy method comprising the step  
9           of administering to a subject an immunostimulatory  
10          amount of hyperimmune globulin prepared according to  
11          Claim 14.

12                   17. A method for the protection against  
13          infection of a subject by enteropathogenic organisms or  
14          hepatitis B virus comprising administering to said  
15          subject an immunogenic amount of an immunostimulating  
16          composition of Claim 3.

17                   18. A method according to Claim 17 wherein the  
18          immunostimulating composition is administered orally.

19                   19. A method according to Claim 17 wherein the  
20          immunostimulating composition is administered  
21          parenterally.

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1                   20. A method according to Claim 17, wherein the  
2 immunostimulating composition is administered in four  
3 separate doses on day 0, day 7, day 14, and day 28.

4                   21. A method according to Claim 17 wherein the  
5           immunogenic substance is the synthetic peptide  
6           representing the peptide fragment beginning with the  
7           amino acid residue 63 through 78 of Pilus Protein CS3  
8           said residue having the amino acid sequence 63(Ser-Lys-  
9           Asn-Gly-Thr-Val-Thr-Try-Ala-His-Glu-Thr-Asn-Asn-Ser-  
10          Ala).

11 PHASE III

In sum, the Colony Factor Antigen (CFA/II) from enterotoxigenic E coli (ETEC) prepared under GMP was successfully incorporated into biodegradable polymer microspheres (CFA/II BPM) under GMP and found to be safe and immunogenic when administered intra-duodenally to rabbits. CFA/II was incorporated into poly (D,L-lactide-co-glycolide) (PLGA) microspheres which were administered by direct endoscopy into the duodenum. Following vaccination, Peyer's patch cells responded by lymphocyte proliferation to in vitro challenge with CFA/II indicating the CFA/II BPM to be immunogenic when administered intra-intestinally. Also, B cells secreting specific anti CFA/II antibodies were found in

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spleens following vaccination. No pathological changes were found following total necropsies of 10 rabbits vaccinated with CFA/II BPM. As a potency test, high serum IgG antibody titers to CFA/II were produced following intra- muscular administration of CFA/II BPM to additional rabbits. The CFA/II BPM contained 63% between 5-10 um by volume particle size distribution; 1.17% protein content; 2.15% moisture; <.01% acetonitrile; 1.6% heptane; 22 nonpathogenic bacteria and 3 fungi per 1 mgm protein dose; and passed the general safety test. We conclude that the CFA/II BPM oral vaccine is immunogenic and safe to begin a Phase I clinical safety study following IND approval.

## INTRODUCTION

Enterotoxigenic Escherichia coli (ETEC) causes diarrheal disease with an estimated 650,000,000 cases annually in developing countries resulting in 500,000 deaths predominantly in the pediatric age groups. Currently there is no vaccine against ETEC induced diarrhea. The availability of an effective oral vaccine would be of great value to the people of South America, Africa and Asia as well as the millions of people who travel to these high risk areas and account for half of the annual cases.



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1           The first step in pathogenesis is adherence to  
2           the small intestine epithelial cells by protein  
3           fimbrial (pilus) adhesins called colonization factor  
4           antigen (CFA). Three major CFAs have been recognized,  
5           CFA/I, CFA/II and CFA/IV. (25)

6           Ten human volunteers who were immunized orally  
7           twice weekly for 4 weeks with CFA/II developed a poor  
8           antibody response and did not show any significant  
9           protection when challenged with pathogenic ETEC (26).  
10          This disappointing response was attributed to adverse  
11          effects of gastric acid, even at neutral pH, of  
12          fimbrial proteins (27). When the vaccine was  
13          administered by inoculation directly into the duodenum,  
14          4 of 5 immunized volunteers developed a significant  
15          rise in secretory IgA with CFA/II antibody (26).

16          D and L-lactic acid and glycolic acid, as homo-  
17          and copolymers, are biodegradable and permit slow and  
18          continued release of antigen with a resultant adjuvant  
19          activity. These polymers have been shown to be safe in  
20          a variety of applications in human beings and in  
21          animals (28-32). Delivery of antigens via microspheres  
22          composed of biodegradable, biocompatible lactide/  
23          glycolide polymers (29-32) may enhance the mucosal  
24          response by protecting the antigen from digestion and

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1 targeting them to lymphoid cells in Peyer's patches  
2 (29-32). McQueen et al. (33) have shown that E coli  
3 AF/R1 pili in PLGA microspheres, introduced intra-  
4 duodenally in rabbits, protected them against diarrhea  
5 and weight loss when challenged with the parent strain  
6 rabbit diarrheagenic strain of E coli (RDEC-1). Only  
7 one vaccinated rabbit of six lost weight and only one  
8 had soft pelleted stool. In contrast, all control  
9 unvaccinated animals became ill, lost weight, and shed  
10 soft pellets or unformed mucoid stool. Significant  
11 lymphocyte proliferation to AF/R1 from Peyer's patches  
12 and ordinary IgA anti AF/R1 antibody levels were seen.

13 In order to improve the CFA/II vaccine it was  
14 incorporated into PLGA microspheres under GMP in order  
15 to protect it from digestion and target it to the  
16 intestinal lymphoid system. The CFA/II BPM vaccine has  
17 undergone pre-clinical evaluation and has been found to  
18 be safe and immunogenic.

#### 19 MATERIALS AND METHODS

20 Preparation of CFA/II Pilus Vaccine. Under Good  
21 Laboratory and Good Manufacturing Practices, E. coli.  
22 strain M424C1-06;816 producing CFA/II were cultured in  
23 75-80 CFA agar plates (24 x 24 cm) for 24 hrs then  
24 harvested by scraping. The harvest was homogenized at

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1        slow speed for 30 minutes with over head drive unit and  
2        cup immersed in an ice bath. The homogenate was  
3        centrifuge at 4° C at 16, 500 x g for 30 minutes. The  
4        supernatant saved and the pellet rehomogenized and  
5        centrifuged with the supernatants pooled. The  
6        supernatant pool was centrifuged at 50,000 x g for 45  
7        minutes. The supernatant treated with ammonium sulfate  
8        at 20% saturation, stirred 30 minutes at 4° C than  
9        stored at 4° C for 16 hrs then centrifuged at 19,700 x  
10       g for 30 minutes. The supernatant saved and treated  
11       with ammonium sulfate at 45% saturation, stirred 30  
12       minutes at 4° C, stored at 4° C for 66-72 hrs, then  
13       centrifuged at 19,700 x g for 45 minutes. The pellet  
14       was resuspended in about 100 mls of PBS containing 0.5%  
15       formalin and held at 22° for 18 hrs then dialyzed for  
16       45-50 hrs against PBS at 4° C using a total of 12  
17       liters in 2 liter amounts. The dialysis was terminated  
18       when the PBS contained less then 0.03% formalin using  
19       Nessler's reagent and fuchsin sulfuose acid reagent.  
20       The final product contained 1 mgm protein/ml PBS, was  
21       sterile and passed the general safety test.

22        Preparation of Desalted CFA/II Vaccine. Two ml  
23        of the CFA/II vaccine were placed into a Centricon 30  
24        tube and centrifuged at 1700 rpm at 4-6° C (Beckman  
25        model GPR centrifuge equipped with GA-24fixed angle  
26        rotor) until all the buffer solution passed through the

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1 filter (about 90-120 minutes). Sterile water was added  
2 to each tube to disperse the CFA/II retained on the  
3 filter. The desalted antigen dispersions from all tube  
4 were pooled and then divided into five equal parts by  
5 weight so as to contain 20 mg of the CFA/II each. The  
6 desalted antigen dispersion was stored at -10 to -20°  
7 C.

8 Freeze Drying of the Desalted CFA/II Dispersion.

9 80 mg of sucrose was added to each part of the CFA/II  
10 dispersion. The resulting mixture was flash-frozen  
11 using a dry ice-acetone bath (100-150 ml of acetone and  
12 50-100 g of dry ice). The frozen solution was freeze  
13 dried overnight using Repp Sublimator 16 freeze dryer  
14 at vacuum of 1 micrometer of mercury and a shelf  
15 temperature not exceeding 37° C.

16 CFA/II Biodegradable Polymer Microspheres.

17 Particle size distribution. About 1 mgm of  
18 microspheres were dispersed in 2 ml of 1% Polysorbate  
19 60° (Ruger Chemical Co. Inc. Irvington, N.J.) in water  
20 in a 5 ml capacity glass vial by sonication. This  
21 dispersion was observed under a calibrated optical  
22 microscope with 43x magnification. Using a  
23 precalibrated eye-piece micrometer, the diameter of 150  
24 randomly chosen microspheres, was determined and the  
25 microsphere size distribution was determined

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1           Scanning Electron Microscopic Analysis.

2           Microspheres were sprinkled on the surface of 10mm stub  
3           covered with a non-conductive adhesive (Sticky-Tab,  
4           Ernest F. Fullem, Inc., Lutham, N.Y.) Samples were  
5           coated with gold/palladium in an automatic sputter-  
6           coating apparatus (Samsputter-2A, Tonsimis Research  
7           Corporation). The samples were examined with a Hitachi  
8           S-450 scanning electron microscope operated at 15-20  
9           KV.

10           Preparation of CFA/II Microspheres. Solvent  
11           extraction technique was used to encapsulate the freeze  
12           dried CFA/II into poly(lactide-co-glycolide) (Medisorb  
13           Technologies International, viscosity 0.73 dl/g)  
14           microspheres in the 1-10 um size range to achieve  
15           theoretical antigen loading of 1% by weight. The  
16           freeze dried antigen-sugar & matrix was dispersed in an  
17           acetolnitrile solution of the polymer and then  
18           emulsified to achieve desired droplet size.  
19           Microspheres were solidified and recovered by using  
20           heptane as extracting solvent. The microsphere batches  
21           were pooled and vacuum dried to remove traces of  
22           solvent.

23           Protein Content. The CFA/II microspheres were  
24           dissolved in 0.9% SDS in 0.1N NaOH for 18 hr with  
25           stirring then neutralized to pH 7 and assayed. The  
26           micro bicichoninic acid (BCA) method was utilized with

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1 both lactic acid and glycolic acid blanks and compared  
2 to bovine serum albumin (BSA) standard and results  
3 expressed as percent by weight.

4 Moisture Content. One hundred and fifty mgm of  
5 CFA/II microspheres were dissolved in 3 ml of  
6 acetonitrile by sonication for 3 hours. One ml sample  
7 was injected into a Karl Ficher titrimeter and triter  
8 reading observed was recorded and acetonitrile blank  
9 was subtracted to determined percent water content.

10 Acetonitrile and Heptane Residuals. Ten mgm of  
11 CFA II microspheres were dissolved in 1 ml DMF then  
12 analysed using gas chromatography and comparing peak  
13 heights to external standards of either acetone or  
14 heptane diluted in DMF with 10 mgm of blank  
15 microspheres. The results are expressed as percent by  
16 weight.

17 Microbial load. One hundred mgm of CFA/II  
18 microsphere(single dose) are suspended in 2 ml of  
19 sterile saline than poured into 2 blood agar plates (1  
20 ml each). All colonies are counted and identified  
21 after 48 hours in culture at 37° C and expressed as  
22 total number. Similiar amount of microspheres is in  
23 0.25 ml aliquots poured onto 4 different fungal culture  
24 plates (Sabharwal, casein peptone agar with  
25 chloramphenicol, brain heart infusion agar with

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1 chloramphenol and genimycin or chloramphenicol alone)  
2 and cultured at 30° for 5 weeks and the colonies  
3 counted & identified and expressed as total number.

4 CFA/II Release From Microsphere Study. Thirty  
5 mgm samples in triplicate were placed in 2 ml conical  
6 upright microcentrifuge tubes containing 1 ml of PBS at  
7 pH 7.4. The tubes were capped and kept immerized in a  
8 water bath maintained at 37° C with constant agitation.  
9 The samples were withdrawn at 1, 3, 6, 8, 15 and 22  
10 hour time intervals by centrifuging the sample tubes  
11 for 5 minutes at the maximum speed of bench top  
12 centrifuge. The release medium was collected through a  
13 5 um nylon screen for CFA/II protein analysis using the  
14 micro BCA method and comparing results to BSA standard  
15 and expressing results as percent cumulative release of  
16 CFA/II.

17 General Safety Test. Two doses of one hundred  
18 mgm CFA/II microspheres were suspended by sonication  
19 for 5 minutes in 3.1 mls of sterile vaccine diluent  
20 consisting of injectable saline containing 0.5%  
21 Polysorbate 60<sup>R</sup> N.F., 0.03 ml were injected  
22 intraperitoneally into each of 2 mice and 3 mls were  
23 administered by gastric lavage to each of 2 guinea  
24 pigs. The animals were weighed both before and at 7  
25 days following the vaccine administration. All animals  
26 were observed daily for any signs of toxicity.

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1           Rabbits. 1.5-2 kilogram male specific pathogen  
2 free New Zealand white rabbits, obtained from closed  
3 colony maintained at the National Institute of Health,  
4 Bethesda, MD. They were selected for study if they did  
5 not have measurable serum antibodies at 1:2 dilution to  
6 CFA/II antigens by ELISA and were not colonized by E.  
7 coli as determined by culture of rectal swabs.

8           Intra-Muscular Immunization of Rabbits and ELISA.

9 Two Rabbits were immunized with CFA/II microsphere  
10 vaccine at 25 ug protein in two different sites intra-  
11 muscularly on day 0. Sera were obtained from all  
12 animals before immunization on day 0 and days 7 and 14.  
13 The sera were tested by ELISA for IgG antibodies to  
14 CFA/II antigen and individual coli surface (CS)  
15 proteins CS3 and CS1. ELISA plates were coated with 3  
16 ug/ml of either CFA/II antigen, CS3 or CS1 protein (150  
17 ul/well) and incubated with 150 ul/well of PBS with  
18 0.1% BSA for four hours at room temperature. The PBS  
19 with 0.1% BSA is washed out with PBS and 100 ul/well of  
20 different dilutions of each rabbit serum in triplicate  
21 was added to the plates. The dilutions ranged from  
22 undiluted to 1:1,000,00. The plates were incubated  
23 with the sera for 3 hours at 37° C. The sera were  
24 washed out with PBS and then horse radish peroxidase-  
25 conjugated goat anti- rabbit IgG was added to the  
26 plates at a 1:1000 dilution (100 ul/well). The plates



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1        were incubated for 1 hour at room temperature with the  
2        peroxidase conjugate. The conjugates were washed out  
3        of the plates with PBS and 100 ul/well of an ABTS  
4        substrate solution (Kikegaard and Perry Laboratories)  
5        was added to each well in the plates. The plates were  
6        read using the ELISA reader (Dynatech Laboratories MR  
7        580) at a wave length of 405 nm after 15 minutes.  
8        The results are measured and expressed as antibody  
9        titers.

10        Intra-duodenal Vaccination of Rabbits. Rabbits  
11        (N=5) were vaccinated with CFA/II microspheres  
12        containing either 25 or 50 ug of protein suspended in 1  
13        ml of PBS containing 0.5% Polysorbate 60<sup>®</sup> on day 0 and  
14        7 by sonication. The microspheres were injected  
15        through an Olympus BF type P10 bronchoscope into the  
16        duodenum of the rabbits following sedation with an  
17        intra muscular injection of ketamine HCl (50 mgm  
18        I.M.) (Ketaset, Fort Dodge Laboratories, Fort Dodge, IA)  
19        and Lylazine (10 mgm I.M.) (Rompom Malay Corporation,  
20        Shnanee, KS). The endoscope was advanced ready under  
21        direct vision into the stomach which was insufflated  
22        with a 50 ml bolus of room air via a catheter passed  
23        through the biopsy channel. The catheter was advanced  
24        through the pylorus 3-4 cm into the duodenum and the  
25        microsphere suspension in 1 ml of PBS was injected,  
26        followed by a 9 ml flush of PBS and removal of the air

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1 bolus. The rabbits were sacrificed by chemical  
2 euthanasia at day 14.

3 Anti-CFA/II Stimulated Lymphocyted

4 Transformation. The Peyer's Patchs were removed and  
5 cell suspension obtained by teasing and irrigation with  
6 a 20 guage needle and syringe. The cells were placed  
7 in 2 ml of media at a concentration of  $2.5 \times 10^6$   
8 cells/ml for each well of a 24 well plate. These cells  
9 were challenged separately with BSA and the CFA/II  
10 antigen at doses of 500, 50 and 5 ng/ml in triplicate  
11 wells. The plates were incubated at 37° C with 5% CO<sub>2</sub>.  
12 On day 4 the cells were mixed while still inside the  
13 wells and 100 ul were transferred into each of 4 wells  
14 in a 96 well flat bottom microculture plate. Thus, the  
15 challenge at each antigen dose represented by 3 wells  
16 in the 24 well plate is now represented by 12 wells in  
17 the 96 well plate. After the cells have been  
18 transferred, each well is pulsed with 20ul of 50 uCi/ml  
19 tritiated thymidine. These pulsed plates were  
20 incubated for 6 hrs then harvester with 96 Mach II Cell  
21 harvested (Tourtec, Inc.). The lymphocyte  
22 proliferation was determined by the tritiated  
23 thymidine incoporation measured in kilo counts per  
24 minute (Kcpm) using the 1205 Beta Plate Liquid  
25 scintillation counter (LKB, Wallac, Inc.). The results

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1 are expressed as mean Kcpm  $\pm$  SD and compared to media  
2 controls.

3 Anti-CFA/II Antibody Secreting B Cells. Spleen  
4 cells were obtained from immunized rabbits on day 14  
5 following intra-duodenal immunization with CFA/II  
6 microsphere vaccine. The cells were placed in 96 well  
7 round bottom microculture plate at a final  
8 concentration of  $6 \times 10^5$  cells/well and incubated for  
9 0, 1, 2, 3, 4 and 5 days at 37° C with 5 CO<sub>2</sub>. 96 well  
10 flat bottom microculture plates were coated with 3  
11 ug/ml of CFA/II antigen overnight blocked with PBS with  
12 0.05% Polysorbate 60<sup>®</sup>. On the harvest days, the cells  
13 were gently flushed out of the wells of the round  
14 bottom plates and transferred to the corresponding well  
15 in the antigen coated, 96 well flat bottom microculture  
16 plates to be tested for the presence of antibody  
17 secreting cells using ELISPOT technique. The plates  
18 were incubated with the cells overnight at 4° C. The  
19 cells were then washed out of the flat bottom plates  
20 with PBS, and 100 ul/well of horserudish-peroxidase  
21 conjugated, goat anti-rabbit total antibody (IgM, IgG,  
22 and IgA) at a 1:1000 dilution were added to the plates.  
23 The Plates were incubated for 1 hour at room  
24 temperature, at which time, the conjugate was washed  
25 out of the plates with PBS. 0.1 mgm of agarose was  
26 dissolved in 10 ml of PBS by boiling. After the agar

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1 solution cooled but not hardened, 6 mgm of 4-chloro-  
2 naphthol, 2 mls of methanol and 30 ul of hydrogen  
3 peroxide were added to make the substrate solution.  
4 The solution was placed into the flat bottom plates  
5 (100 ul/well) and the plates were held at 4°C overnight  
6 so the agar could harden. The number of browish spots  
7 per 15 wells (total of  $9 \times 10^6$  spleen cells) was  
8 counted and represents the number of antibody secreting  
9 cells per  $9 \times 10^6$  spleen cells.

10 Pathological Evaluation. Rabbits were euthanized  
11 by parenteral overdose of sodium pentobarbital and were  
12 subjected to complete necropsy. Sample of tissue  
13 including small and large intestine with gut associated  
14 lymphoid tissue, spleen, mesenteric and mediastinal  
15 lymph nodes, lung, trachea, liver and kidney were fixed  
16 by immersion in 10% neutral buffered formalin. Tissues  
17 were routinely processed for light microscopy and  
18 embedded in paraffin. Five micron thick sections were  
19 stained with hematoxylin and eosin.

20 Statistical Analysis. The paired student t-test  
21 was used to determine p values.

## 22 RESULTS

23 Particle Size Distribution. The results of size  
24 frequency analysis of 150 randomly chosen microspheres  
25 are shown in (Figure 29). The particle size

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1 distribution is plotted in % frequency against particle  
2 size in diameter (size) expressed in um. The average  
3 number frequency diameter is 4.6 um. The average volume  
4 frequency diameter is 4.6 um. The percent volume  
5 between diameters of 5-10 um is 63% and the percent  
6 volume less than 10um diameter is 88%.

7 Scanning Electron Microscopy. The microspheres  
8 are seen in (Figure 30) which is a scanning electron  
9 photomicrograph. Nearly all the microspheres are less  
10 than 10 um as compared to the 5 um bar. Also the  
11 surfaces of the microsphere are smooth and demonstrate  
12 lack of pores.

13 Protein Content. The protein loads of the  
14 individual batches are the following: K62A8, 1.16%  $\pm$   
15 0.10 SD; K63A8, 1.023%  $\pm$  0.17SD; K64A8, 1.232%  $\pm$  0.13  
16 SD; and K65A8, 0.966%  $\pm$  0.128 SD. The mean average  
17 protein load is 1.16%  $\pm$  0.15 SD. The protein load of  
18 the CFA/II microsphere vaccine in the final dose vial  
19 is the following: Lot L74F2, 1.175%  $\pm$  0.17SD.

20 Moisture Content. The CFA/II microsphere vaccine  
21 (Lot 74F2) percent water content was found using the  
22 Karl Fischer titrimeter method to be 2.154% using  
23 triplicate samples.

24 Acetonitrile and Heptane Residuals. The  
25 acetonitrile residuals of the 4 individual CFA/II  
26 microsphere batches are the following: K62A8, <0.1%;

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1 K62A8, <0.1%, K64A8, <0.1%; and K65A8, <0.1%. The  
2 acetonitrile residual of the CFA/II microsphere vaccine  
3 in the final dose vial is the following: Lot L74F2,  
4  $0.07 \pm 0.05\%$ . The heptane residual of the 4 individual  
5 CFA/II microsphere batches are the following: K62A8,  
6 1.9%; K63A8, 1.4%; K64A8, 1.6% and K65A8, 1.6%.  
7 Following pooling in heptane and subsequent drying, the  
8 heptane residual of the CFA/II microsphere vaccine in  
9 the final dose vial is the following: Lot L74F2,  $1.6 \pm$   
10  $0.1\%$ .

11 Microbial load. One hundred milligrams (a single  
12 dose) of CFA/II microsphere vaccine (Lot L74F2) in the  
13 final dose vial was suspended in a 2 ml of sterile  
14 saline and 1 ml poured onto a blood agar culture plate  
15 x 2. Twenty two colonies grew after 48 hours of  
16 culture and 21 were identified as coagulase negative  
17 staphylococcus and 1 as a micrococcus species. All these  
18 bacteria are considered to be nonpathogenic to humans.  
19 An additional 100 mgms of CFA/II microsphere vaccine  
20 (Lot L74F2) were suspended in 2 ml of sterile saline  
21 and 0.25 ml poured onto four different fungal culture  
22 agars and cultered for 5 weeks. Three fungal colonies  
23 grew and each was identified as A. glaucus.

24 CFA Release From Microsphere Study. Three thirty  
25 mgm samples were incubated each in 1 ml of PBS, pH 7.4

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1 at 37° C for 0, 1, 3, 6, 8, 15 and 22 hours. The  
2 supernates were removed and replaced at these times.  
3 The protein content was determined for each supernate  
4 sample and the results are seen in (Figure # 31). The  
5 results are plotted as percent release of CFA/II  
6 against time in hours. An average of 8% of CFA/II is  
7 released at one hour rising to 20% at 8 hours then a  
8 slower release to 25% at 22 hours.

9 General Safety Test. Two one hundred milligrams  
10 (a single dose) of CFA/II microsphere vaccine in the  
11 final dose vials were suspended in 3.1 mls of the  
12 sterile diluent consisting of 0.85 N saline prepared  
13 for injection plus Polysorbate 60<sup>R</sup> at 0.5%. Two Swiss  
14 mice (16.5 gm) were injected intraperitoneally with  
15 0.03 mls and two Hartley guinea pigs (350 gm) were  
16 administered by gastric lavage 3.0 mls.

17 None of these animals displayed any signs of  
18 toxicity for 7 days. The mice gained an average of  
19 2.3 gms and the guinea pigs gained an average, of 43  
20 grams. The CFA/II microsphere vaccine therefore  
21 passed the general safety test.

22 Serum IgG Antibody Responses. Two rabbits were  
23 immunized in two separate sites intra-muscularly with  
24 25 ug of protein of CFA/II microsphere vaccine (Lot  
25 L74F2) in the final dose vial. Sera samples were  
26 obtained before and 7 and 14 days following

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1 immunization. The IgG antibody titers to CFA/II CSI  
2 and CS3 protein were determined using ELISA and the  
3 results seen in (Figure 32). The results are expressed  
4 as mean antibody titers against the different antigens  
5 at 0, 7 and 14 days. High antibody titers greater than  
6 1000 were seen at 7 days to both CS1 and CS3 protein  
7 which rose to greater than 10,000 by day 14. The  
8 individuals titers to CFA/II are seen in (Figure 33).  
9 Rabbit 109 developed an antibody titer of 1,000 by day  
10 7 rising to 3,000 by day 14. Rabbit 108 had a log  
11 higher rise at day 7 and 2 log higher rise at day 14  
12 being  $3 \times 10^4$  at day 7 going to  $1 \times 10^5$  at day 14.

13 Anti-CFA/II Stimulated Lymphocyte Transformation.

14 Five rabbits were immunized intra-duodenally with  
15 CFA/II microspheres containing either 25 ug of protein  
16 (human dose equivalent) or 50 ug of protein on days 0  
17 and 7 and then sacrificed on day 14. The Peyer's patch  
18 lymphocytes were challenged in vitro with CFA/II  
19 antigen, BSA media and alone. The lymphocyte  
20 transformation was determined by tritiated thymidine  
21 incorporation. The results of the high dose  
22 immunization are seen in (Figure 34). The results are  
23 expressed as Kcpm against antigen dose. No response to  
24 BSA or media control is seen in any of the five  
25 rabbits. All rabbits responded by lymphocyte



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1 transformation in a dose dependent manner to the  
2 CFA/II.

3 The highest dose responses were 3-10X's the media  
4 control are highly significant with a p value of  
5 <0.002. The results of the 5 rabbits receiving the low  
6 dose immunization are seen in (Figures 35). Rabbit #80  
7 gave no response probably due to poor Peyer's patch  
8 cell population which did not respond were to  
9 Conconavallin A mitogenic stimulation either. The  
10 remaining 4 rabbits gave positive responses with the  
11 high CFA/II dose response being 2-8x media control and  
12 highly significant with p values of <0.009. Again no  
13 response were seen to BSA compared to the media cont

14 Anti-CFA/II Antibody Secreting B-Cells Five  
15 rabbits immunized intraduodenally with CFA/II  
16 microsphere containing 50 ug of CFA/II protein at days  
17 0, 7 than sacrificed at day 14 were studied. The spleen  
18 cells were placed into microculture then ELISPOT  
19 forming B-Cells secreting specific anti CFA/II antibody  
20 determined at days 0, 1, 2, 3, 4 and 5. The results  
21 are seen in (Figure 36) and expressed as # of antibody  
22 secreting cells per  $9 \times 10^6$  spleen cell against culture  
23 days. Positive responses were seen in all 5 rabbits on  
24 days 2-5. Days of maximum responses occurred on day 3  
25 for rabbits 65 and 66; day 4 for rabbit 85; and day 5  
26 for rabbits 83 and 86. The responses are highly

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1 significant being 7-115 times higher than the 1-2 cells  
2 seen on all days in 4 control rabbit (67, 69, 72, 89)  
3 (Figure 37). Here is a composite graph expressing the  
4 mean counts  $\pm$  ISD for all days of culture.

5 Pathological Evaluation. A consistent finding in  
6 the spleens of all rabbits both the 25 and 50 ug  
7 protein dose groups was minimal to mild diffuse  
8 lymphocytic hyperplasia the periarteriolar lymphatic  
9 sheaths (T cell dependent areas). Two of five rabbits  
10 of the 50 ug dose group (#83 and #86) also had mild  
11 lymphocytic hyperplasia of splenic follicular (B cell  
12 dependent) areas. The three rabbits in an untreated  
13 control group had histologically normal spleens.

14 Reactive hyperplasia of mesenteric lymph nodes  
15 was often seen in vaccinated rabbits. Two of five  
16 rabbits in the 25 ug dose equivalent group (#83 and  
17 #86) also had minimal to mild lymphocytic hyperplasia  
18 of cortical follicular (B cell dependent) areas. The  
19 mesenteric lymph nodes of the other vaccinated rabbits  
20 and of the untreated control rabbits were within normal  
21 limits. Incidental or background lesions found in one  
22 or more rabbits of all three group were acute minimal  
23 to mild pneumonia and foreign body microgranulomas of  
24 the cecal gut associated lymphoid tissue.

25 Discussion

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1 McQueen et al (33) has found that the AF/R1 adhesin of  
2 rabbit diarrheagenic Escherichia coli (RDEC-1)  
3 incorporated into biodegradable microspheres could  
4 function as a safe and effective oral intestinal  
5 vaccine in the rabbit diarrhea model. The AF/R1 was  
6 incorporated into poly D,L-lactide-co-glycolide)  
7 microspheres and administered intraduodenally. Jarboe  
8 et al (34) reported that

9 Peyer's patch cells obtained from rabbits  
10 immunized intra-duodenally with AF/R1 in microspheres  
11 responded with lymphocyte proliferation upon in vitro  
12 challenge with AF/R1. This early response at 14 days  
13 gave a clear indication as to the immunogenicity of E.  
14 coli pili contained within the polymer microspheres.

15 In developing an effective oral vaccine against  
16 enterotoxigenic E. coli, CFA/II pili given as an oral  
17 vaccine was found to be ineffective. The CFA/II pilus  
18 proteins were found to be rapidly degraded when treated  
19 with 0.1M HCl and pepsin conditions mimicking those  
20 contained in the stomach (27). The CFA/II was found to  
21 be immunogenic when given in high doses intra-  
22 intestinally producing intestinal secretory IgA  
23 antibodies (26).

24 The CFA/II vaccine has now been incorporated into  
25 poly(D,L lactide-co-glycolide) microspheres under Good  
26 Manufacturing Practices and tested under Good

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1 Laboratory Practices. The microspheres, are spherical,  
2 smooth surfaced and without pores. The majority (63%)  
3 are between 5-10 um in diameter by volume. This size  
4 range has been suggested to promote localization within  
5 the Peyer's patch in mice and perhaps enhance local  
6 immunization (29-32). The protein content being 1.174%  
7 is close to 1% which was the goal of the vaccine  
8 formulation. One percent was chosen because 0.62% was  
9 the core loading of the AF/R1 microspheres which were  
10 effective. Also a small precentage perhaps 1-5% (35)  
11 is anticipated to be taken up from the intestine, a  
12 higher protein content would lead to considerable loss  
13 of protein.

14 The organic residuals are of course a concern.  
15 Heptane exposure would be 1.7 mgm per vaccine dose.  
16 This is compared to the occupational maximum allowable  
17 exposure of 1800 mgm/15 min. Therefore, the heptane  
18 contained with the CFA/II microsphere vaccine appears  
19 to be a safe level. The acetonitrile is very low - 0.1  
20 mgm per vaccine dose. The human oral TDLO is 570  
21 mgm/Kg (any non letheal toxicity). Therefore, the  
22 acetonitrile contained with the CFA/II microsphere  
23 vaccine appears to be at a safe level. The CFA/II  
24 vaccine was produced under sterile conditions.  
25 However, the process of incorporation of the desalted  
26 CFA/II vaccine into the polymer microsphere batches and

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1 subsequent pooling and loading final dose vials was  
2 done in a clean room as for any oral medication. It  
3 was expected and found that there was be a microbial  
4 load. The guide used was the World Health Organization  
5 (WHO) Requirements of Thyphoid Vaccine (Live  
6 Atttaruated, Ty 21a oral). Two hundred non pathogenic  
7 bacteria are allowed as well as 20 fungi per dose. The  
8 CFA/II microsphere vaccine is well under these  
9 requirements having only 22 non-pathogenic bacteria and  
10 3 fungi per dose.

11 The general safety test was also patterned after  
12 the WHO requiremets for the TY, 21a oral vaccine in  
13 that the CFA/II microsphere vaccine was give by gastric  
14 lavage to the guinea pigs. Both mice and both guinea  
15 pigs demonstrated no toxicity & gained weight over the  
16 7 day test clearly indiciating the innoccuos nature of  
17 this vaccine by passing this safety test.

18 The CFA/II microsphere vaccine (Lot74F2) is  
19 immunogenic giving high titer serum IgG antibody  
20 responses as early as 7 days following intra muscular  
21 injection in rabbits. This test will be used as  
22 potency test for future lots of the CFA/II microsphere  
23 vaccine. Slightly higher antibody titers were seen  
24 towards the CS3 pilus protein and this may reflect that  
25 CS3 accounts for 90% of the protein in the CFA/II and  
26 CS1 10% (36).

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1           The CFA/II microsphere vaccine was also  
2           immunogenic following intra-duodenal administration to  
3           rabbits. The highest lymphocyte proliferative  
4           responses from Peyer's patch cells were seen with the  
5           lower 25 ug dose. This is the human equivalent dose  
6           and suggests that higher doses of antigen in polymer  
7           microspheres may attenuate, this immunological response.

8           The antibody secreting B-cells demonstrated in  
9           the rabbit spleen at 14 days is a clear indication that  
10          B-cells have been immunized. They may represent  
11          resident B-cells immunized in the spleen or B-cells  
12          immunized at the level of the Peyer's patches and are  
13          migrating through the spleen to return to the  
14          intestinal mucosal lamina propria (1-3). The delay of  
15          several days before secreted antibody is detected  
16          suggests either maturation is required of the B-cells  
17          or that down regulation may be present initially and  
18          lost with time in culture.

19          Further evidence of immunization by the CFA/II  
20          microsphere vaccine given intra-duodenally is  
21          demonstrated by the lymphatic hyperplasia in the spleen  
22          seen to a greater extent in the rabbits receiving the  
23          lower dose 5/5 compared to 2/5 of the rabbits receiving  
24          the higher 50 ug protein dose. On the other hand,  
25          greater T-cell dependent area lymphocytic hyperplasia in  
26          the mesenteric lymph nodes were seen in rabbits

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1 receiving the higher 50 ug dose, 4/5 compared to 2/5.  
2 These changes are most likely due to the vaccine since  
3 similar changes were not seen in three untreated  
4 control rabbits. Also no abnormal pathological changes  
5 attributable to the vaccine were seen.

6 The CFA/II BPM vaccine has undergone pre-clinical  
7 evaluation and has been found safe and immunogenic.  
8 This vaccine is ready for clinical Phase I safety  
9 testing following FDA's IND approval.

10 PHASE IV

11 In sum, alum precipitation, vaccination regimen  
12 and controlled delivery by microencapsulation were  
13 studied to determine what criteria must be satisfied to  
14 provide a protective immune response to hepatitis B  
15 surface antigen (HBsAg) after a single injection of  
16 vaccine. In mouse studies, the 50% effective dose  
17 (ED<sub>50</sub>) for the alum precipitated Heptavax B vaccine  
18 (Merck, Sharp and Dohme) was 3.8 ng when administered  
19 in a 3 injection regimen, but was 130 ng when one  
20 immunizing dose was used. Antigen release studies  
21 revealed that HBsAg is bound tightly to the alum,  
22 indicating that the antigen remains in situ until  
23 scavenged by phagocytic cells. the ED<sub>50</sub> with a 3 dose  
24 regimen of aqueous HBsAg was 180 ng, as opposed to over  
25 2000 ng for daily injections of low doses for 90 days  
26 and 240 ng for a regimen that employed initially high

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1 doses that decreased geometrically at 3 day intervals  
2 over 90 days. The ED<sub>50</sub> was 220 ng for a single dose  
3 regimen of HBsAg microencapsulated in poly (DL-lactide-  
4 co-glycolide) in a form that was too large to be  
5 phagocytized and had an antigen release profile similar  
6 to that achieved with the geometrically decreasing  
7 regimen of doses. This indicates that single injection  
8 of microencapsulated immunogens can achieve similar  
9 effects in vivo to those achieved with multiple dose  
10 regimens. For HBsAg the effect to be achieved appears  
11 to be 3 pulses of particulate immunogens that can be  
12 scavenged by phagocytes.

#### 13 INTRODUCTION

14 A major disadvantage of inactivated vaccines  
15 lies in their inability to confer lasting immunity.  
16 Due to rapid elimination from the body, multiple doses  
17 and boosters are usually required for continued  
18 protection<sup>37</sup>. Alum adjuvants, achieving their effects  
19 by mechanisms of antigen presentation and sustained  
20 antigen release<sup>38</sup>, have been used successfully to  
21 increase the potency of several inactivated vaccines  
22 including those against tetanus, anthrax, and serum  
23 hepatitis<sup>39,40</sup>. Though useful, alum preparations are  
24 deficient in several aspects. Control over quantity  
25 and rate of antigen release is limited, often resulting  
26 in a continued requirement for immunization schedules



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1       consisting of multiple injections given over a period  
2       of several months to years. Alum adjuvants are also  
3       non-biodegradable and thus remain within the body,  
4       serving as a nidus for scar tissue formation<sup>38</sup> long  
5       after they have served their function.

6               Protracted, multiple immunization schedules are  
7       unacceptable during massive mobilization and deployment  
8       of troops. Changing global disease patterns and  
9       deployment of new biological warfare agents by enemy  
10      forces require flexibility in the number and types of  
11      vaccine antigen administered to soldiers departing for  
12      combat. Any immunization schedule requiring completion  
13      during engagement in non-linear combat would compromise  
14      this flexibility and place an unreasonable burden on  
15      our health care delivery system.

16             The main objective of this study was, therefore,  
17      to develop a biodegradable, controlled-release adjuvant  
18      system capable of eliminating the need for multistep  
19      vaccination schedules. This investigation was designed  
20      to : (1) determine in an animal model hepatitis B  
21      vaccine release rate characteristics desirable for  
22      single-step immunization, (2) incorporate those release  
23      rate characteristics into a one-step biodegradable  
24      poly(DL-lactide-co-glycolide) (DL-PLG) microencapsulated  
25      hepatitis B surface antigen (HBsAg) vaccine, and (3)  
26      conduct an in vivo trial comparing the effectiveness of

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1       this single-step vaccine against the conventional  
2       three-step hepatitis vaccine currently employed<sup>41</sup>. The  
3       results were intended to provide the foundation for  
4       further development of single step vaccines against  
5       hepatitis and other militarily significant diseases<sup>42</sup>.

#### 6                   MATERIALS AND METHODS

7               Vaccine potency assay. Due to its availability,  
8       compatibility with cage mates, and potential  
9       application to the study of hepatitis B vaccine<sup>43</sup>, the  
10      female Walter Reed (ICR) stain mouse was used. A  
11      hepatitis B vaccine potency assay for comparing the  
12      six-month immunization schedule currently in use<sup>41</sup> with  
13      that of a single-step immunization by sustained antigen  
14      release was established according to the following  
15      protocol: Specimens for baseline antibody titers were  
16      collected from twenty mice by exsanguination.  
17      Immediately prior to exsanguination, all mice employed  
18      in this and other exsanguination procedures in these  
19      studies were anesthetized with a 0.1 ml injection of V-  
20      Pento. Groups of 12 mice were then immunized according  
21      to a schedule consisting of either 0.25 ug, 0.025 ug,  
22      2.5 ng, 0.25 ng, 2.5 pg, or 0.25 pg Heptavax B vaccine  
23      (HBV) administered in 50 microliter volumes  
24      subcutaneously (s.c.) at the beginning and end of the  
25      first, and end of the second month of the protocol.  
26      Antibody responses to the vaccine were monitored

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1 immediately before the third injection and  
2 approximately one month after the third injection.  
3 Specimens for antibody determination were collected by  
4 exsanguination of seven anesthetized mice from each  
5 group and assayed along with the baseline samples by  
6 the Abbott Ausab radioimmunoassay. Percent  
7 seroconversion verses micrograms vaccine employed with  
8 calculated by the method of Reed and Muench<sup>43</sup>. These  
9 data were employed to establish a mouse vaccine potency  
10 assay calibrated to detect differences between Heptavax  
11 B and other forms of hepatitis b vaccine.

12 In vitro antigen release rate from Heptavax B  
13 vaccine. Antigen release from aluminum hydroxide  
14 adjuvant in HBV was measured by pumping 2 cc per hour  
15 of 1:20,000 thimerosal in saline at 4°C across a 0.2 µ  
16 pore diameter Acrodisc filter apparatus containing 20  
17 µg of vaccine. The effluent, collected by a Gilford  
18 fraction collector, was assayed periodically over  
19 several weeks for protein by UV absorption at 280 nm on  
20 a Beckman model 25 double beam spectrophotometer, and  
21 for HBsAg by the Abbot Ausria II radioimmunoassay made  
22 quantitative by using HBsAg standards supplied by Merk,  
23 Sharp, and Dohme. Accuracy of the HBsAg standards were  
24 verified by Biuret protein determination and by UV  
25 absorbance at 215 nm and 225 nm<sup>44</sup>. Nonspecific antigen  
26 retention on the Acrodisc filter was assessed by

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1 measuring percent recovery of a known quantity of  
2 HBsAg. Spontaneous degradation of vaccine antigen was  
3 monitored by comparing daily rations of antigen to  
4 total protein detected in the effluent.

5 Evaluation of HBsAg stability. These studies  
6 were designed to characterize the stability of the  
7 aqueous antigen to the various physical conditions  
8 employed in the microencapsulation process. Conditions  
9 tested included lyophilization with reconstitution in  
10 distilled water, cyclohexane, methylene chloride,  
11 chloroform, methyl alcohol, acetone, iso-octane,  
12 hexane, acetone, pentane, or heptane; irradiation while  
13 lyophilized; and, exposure to elevated temperatures.  
14 Samples exposed to organic solvents were first  
15 lyophilized, reconstituted with the test solvent,  
16 evaporated to dryness under nitrogen at room  
17 temperature and reconstituted with distilled water.  
18 Test samples were compared against untreated controls  
19 by assaying serial dilutions of each with the Abbot  
20 Ausria II procedure and comparing the plots of counts  
21 per minute verses dilution.

22 Assessment of the effect of antigen release rate  
23 on vaccine potency. Three regimens simulating patterns  
24 of free HBsAg release that could be achieved by  
25 microencapsulation were contrasted with the three  
26 monthly dose regimen of Heptavax B for immunizing mice.

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1 To do so, 24 ICR mice were divided into groups and  
2 vaccinated as indicated below. Seven mice from each  
3 subgroup were exsanguinated at the end of the second  
4 and third months of the experiment. The sera were  
5 separated and assayed for specific antibody response to  
6 HBsAg by Abbot Ausab procedure.

7 HV regimen a: 14 mice/treatment receiving 3 s.c.  
8 injections of 250, 25, 2.5 or 0.25 ng doses of HBV a  
9 month apart.

10 HBsAg regimen a: 14 mice/treatment receiving 3  
11 s.c. injections of 250, 25, 2.5 or 0.25 ng doses of  
12 aqueous HBsAg a month apart.

13 HBsAg regimen b: 14 mice/treatment receiving  
14 total doses of 750, 75, 7.5 or 0.75 ng of aqueous HBsAg  
15 over 3 months by s.c. injections of  $ZX_y$  ng at 3 day  
16 intervals, where Z is the total dose, y is the  
17 injection number, and X is the fraction indicated on  
18 the graph in Fig. 1 minus the fraction for the previous  
19 injection.

20 HBsAg regimen c: 14 mice/treatment receiving  
21 daily s.c. injections of 8.33, 0.833, 0.0833 or 0.00833  
22 ng of aqueous HBsAg for 3 months.

23 Microencapsulation in DL:PLG. Microencapsulated  
24 immunogens were fabricated by Southern Research  
25 Institute, Birmingham, AL. DL-PLG polymers were  
26 synthesized from the cyclic diesters, DL lactide and

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1 glycolide, by using a ring-opening melt polymerization  
2 catalyzed by tetraphenyl tin<sup>45</sup>. The resulting polymer  
3 was dissolved in methylene chloride, filtered free of  
4 insoluble contaminants and precipitated in methanol.  
5 Lactide-co-glycolide mole ratio of the product was  
6 determined by nuclear magnetic resonance spectroscopy.  
7 Encapsulation of HBsAg in DL:PLG polymer was achieved  
8 by an organic phase separation process<sup>46</sup>. Microcapsules  
9 of the desired size (approximately 100 micron diameter  
10 in these studies) were isolated from each batch by wet  
11 sieving with hexane through standard mesh stainless  
12 steel sieves and then dried for 24 hours in a vacuum  
13 chamber maintained at room temperature.

14 In vitro analysis of encapsulated antigens.

15 Integrity of encapsulated antigen was assessed by  
16 comparing the antigen to total protein ratios present  
17 in microcapsule hydrolysates with those obtained from  
18 suspensions of pure unencapsulated antigen. Centrifuge  
19 tubes containing 1 ug of either microencapsulated or  
20 pure vaccine antigen in 1 ml saline were incubated at  
21 4°C with shaking. Samples were collected at weekly  
22 intervals by interrupting the incubation, sedimenting  
23 the contents of the tubes by centrifugation and  
24 withdrawing the supernates. Sediments were resuspended  
25 in 200 microliters of saline and supernates were  
26 assayed for HBsAg by the Abbott Ausria II

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1 radioimmunoassay. The HBsAg standard described earlier  
2 in this report was used as the calibrator. Antigen  
3 destruction due to the encapsulation procedure was  
4 monitored by a comparison between the antigen assayed  
5 from the hydrolysate and from the untreated antigen  
6 control.

7 Assessment of the potency of DL:PLG  
8 microencapsulated HBsAg for immunizing ICR mice when  
9 used alone and in combination with Heptavax B vaccine.  
10 HBsAg loaded microcapsules that had been fabricated by  
11 Southern Research Institute to release the majority of  
12 their HBsAg load within 40 to 50 days were serially  
13 diluted in 10-fold steps by mixing the dry, loaded  
14 capsules with blank placebo capsules of similar size  
15 and composition. The resulting stock and diluted  
16 microcapsule preparations were placed onto lyophilizer  
17 when not in use in order to assure minimum spontaneous  
18 degradation prior to injection. On the day of  
19 injection, a predetermined weight of microcapsules or  
20 placebo-diluted microcapsules was added to each  
21 syringe. Immediately prior to injection either one or  
22 two ml of injection vehicle (2 wt % carboxymethyl  
23 cellulose and 1 wt % Tween 240 in water, Southern  
24 Research Institute) were drawn into the microcapsule-  
25 loaded syringes, mixed and injected. All mice were  
26 vaccinated s.c. as indicated below:

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1           Group 1: 14 mice/treatment receiving 25, 25, 2.5,  
2           0.25 or 0.925 ng HBV.

3           Group 2: 14 mice/treatment receiving 1000, 250,  
4           25 or 2.5 ng aqueous HBsAg with Bovine Serum Albumin  
5           (BSA).

6           Group 3: 7 mice receiving 1600 ng  
7           microencapsulated HBsAg (HBsAg) plus 0.25 ng HBV and 14  
8           mice/treatment receiving 160, 16, 1.6 or 0.16 ng HBsAg  
9           plus 0.25 ng HBV.

10          Group 4: 7 mice receiving 1600 ng HBsAg plus 2.5  
11          ng HBV and 14 mice/treatment receiving 160, 16, 1.6 or  
12          0.16 ng HBsAg plus 2.5 ng HBV.

13          Group 5.: 7 mice receiving 1600 ng HBsAg plus 25  
14          ng HBV and 14 mice/treatment receiving 160, 16, 1.6 or  
15          0.16 ng HBsAg plus 25 ng NBV.

16          Group 6: 7 mice receiving 2500 ng HBsAg and 14  
17          mice-treatment receiving 250, 25, 2.5 or 0.25 ng HBsAg.  
18          Fifty-three days after receiving the above injections,  
19          the mice were anesthetized with an 0.1 cc injection of  
20          V-Pento and exsanguinated. Blood samples were allowed  
21          clot and the sera were separated by centrifugation.  
22          The serum samples were assayed for antibody to HBsAg by  
23          the Abbott Ausab procedure.

24

## RESULTS



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1                    Heptavax B vaccine potency. As can be seen from  
 2                    Table 4, the total dose of vaccine which produced  
 3                    seroconversion in 50% of

4                    TABLE 4. Potency of Heptavax B vaccine in ICR mice.

No.	ng Heptavax B per Injection							
	ED <sub>50</sub>							
Inj.	250	25	2.5	.25	.025	.0025	.00025	
ng								
2	5/5	4/4	3/6	2/6	0/5	1/4	0/4	
1.7								
3	6/6	6/6	4/6	1/6	0/6	1/6	1/6	
2.0								

16                    \* Number positive seroconversions per number  
 17                    vaccinated.

18                    The vaccinated mice (ED<sub>50</sub>) for HBV was approximately 2  
 19                    ng, whether the vaccine was given in 2 or 3 injections.

20                    In vitro antigen release rate from HBV. HBsAg  
 21                    release from the 20 ug of Heptavax was not detected in  
 22                    any of the 21 fractions of saline collected from the  
 23                    Acrodisc polycarbonate filter over a 30 day period.  
 24                    The lower limit of detection for the Abbott Auria II  
 25                    assay employed was approximately 4.8 ng/ml. The

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1       Acrodisc filter used in the antigen release study was  
2       back-washed with 10 mls normal saline. Quantitation of  
3       the HBsAg present within this back-wash eluent revealed  
4       the presence of the original 40 ug of Heptavax vaccine  
5       which had been loaded into the filter at the start of  
6       the experiment. This is the concentration which one  
7       would expect to obtain if there had been no  
8       deterioration of the original 40 ug/ml HBsAg loaded  
9       onto the filter, none of the antigen eluted from the  
10      alum adjuvant, and none of the vaccine had adsorbed  
11      onto or passed through the filter.

12           Evaluation of antigen stability. Considerable  
13      effort was expended in assessing the effects of  
14      physical conditions on the antigenicity of HBsAg to  
15      insure that the conditions used for microencapsulation  
16      would not cause serious degradation of the immunogen.  
17      Since microencapsulation must be performed on dried  
18      materials which are suspended in organic solvents, the  
19      HBsAg, which was provided as a solution, had to be  
20      lyophilized. Initial attempts at lyophilizing HBsAg in  
21      normal saline resulted in a total loss of detectable  
22      antigen within samples. Dilution of the HBsAg sample  
23      1:10 in distilled water prior to freezing resulted in  
24      reservation of nearly 100% of the antigen detectable in  
25      the original sample. Studies of antigen stability at  
26      elevated temperature revealed that HBsAg may be heated

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1 to 50°C for up to one hour without appreciable loss of  
2 antigen. The studies involving exposure of lyophilized  
3 antigen to organic solvents indicated that iso-octane and  
4 hexane had minimal effects on antigenicity, but that  
5 95% to 100% of antigenicity was lost upon exposure to  
6 either methylene chloride, chloroform, cyclohexane, or  
7 methyl alcohol. Moderate antigen loss occurred in the  
8 presence of acetone, pentane and heptane. As a result  
9 of these studies, hexane was chosen as the solvent for  
10 microencapsulation.

11 Assessment of the effect of antigen release rate  
12 on vaccine potency. The results (Table 5) indicated  
13 that immunogen formation (i.e., the alum adjuvant of  
14 Heptavax B) had far more

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TABLE 5. Effect of immunogen formulation and vaccination regimen on potency for immunizing ICR mice.

Immunogen		ng Total Dose HBsAg					ED <sub>50</sub>
Formulation	Regiment	750	75	7.5	.75	ng	
Heptavax B	a	7/7*	6/6	5/7	1/7		3.8
Aqu. HBsAg	a	4/6	3/7	0/7	0/6		180
Aqu. HBsAg	b	6/7	0/7	1/7	0/7		240
Aqu. HBsAg	c	1/7	0/7	0/7	0/7		>2000

\* Number positive seroconversions per number vaccinated.

a 3 injections of 1/3 total dose a month apart.

b Injections administered every three days for 90 days in decreasing dosages according to a logarithmic progression.

c Injections of 1/90 total dose daily for 90 days.

effect on potency than did the vaccination regimen, and that pulsing with large doses of immunogen was more effective than continuous administration of small doses.

HBsAg release from DL:PLG microcapsules. The microcapsules employed in this study were designed to

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disintegrate within three weeks after hydration. It is evident from the release curve (Fig. 2) that they performed as designed, releasing approximately 17% of their total load in an initial pulse and approximately 7% of the remaining available HBsAg over the first three weeks.

Assessment of the potency of DL:PLG

microencapsulated HBsAg for immunizing ICR mice when used alone and in combination with Heptavax B vaccine.

The results (Table 6) indicate that the microencapsulated HBsAg had approximately the same immunogenicity as did the Heptavax B. Neither immunogens were sufficiently potent to effect with a singly injection seroconversion rates similar to those achieved after three injections of Heptavax B (Table 4). Only the immunogen

TABLE 6. Potencies of Heptavax B and microencapsulated HBsAg by single injection S.C. when administered alone and in combination to immunize ICR mice.

Var. Dose	ng Const.	ng Variable Dose				Var. Dose Tot.	
Dose							
Immunogen Dose	mHBsAg	2500	250	25	2.5	.25	ED <sub>50</sub> ng
ED <sub>50</sub>	ng						

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1	Heptavax B	0	13/14*	8/14	4/14	0/13	130
2	130						
3	Heptavax B	0.16		11/13	4/14	1/14	1.7
4	1.8						
5	Heptavax B	1.6		10/13	1/14	0/13	100
6	100						
7	Heptavax B	16		3/14	1/14	1/14	>470
8	>490						
9	Heptavax B	160		3/12	2/11	1/12	>370
10	>530						
11	Heptavax B	1600		7/7	7/7	7/7	<0.8
12	1600						
13	Mic. HBsAg	0	3/6	6/15	1/13	2/10	2/14 220
14	220						

15

16

17 \* Number positive seroconversions per number vaccinated.

18 combination of Heptavax B with 0.16 ng mHGSAg provided  
 19 this level of seroconversion. At the ED<sub>50</sub> endpoint, the  
 20 0.16 ng dose of mHGSAg is approximately 10% of the  
 21 total dose. Similarly, a small amount of Heptavax B  
 22 appeared to enhance the immunogenicity of the  
 23 microencapsulated immunogen, although the combination  
 24 was clearly less immunogenic when the two formulations  
 25 were present at equivalent concentrations.

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TABLE 7. ANTIBODY SECRETING CELL RESPONSES TO CFA/II VACCINE BY ELISPOT ASSAY  
AFTER VACCINATION WITH CFA/II ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS  
0, 7, 14, AND 28 (E. COLI CVD 15001)

	vaccinee	IgA				IgG				IgM			
		pre	+7	+14	+21	+35	pre	+7	+14	+21	+35	pre	+7
6	15001-1	0	0	0	0	0	0	0	0	0	0	0	0
7	15001-2	0	0	0	0	0	0	0	0	0	0	0	0
8	15001-3'	0	22	0	0	10	0	0	0	0	0	0	0
9	15001-4	0	6	0	0	16	0	0	0	0	0	0	0
10	15001-6'	0	0	0	0	0	0	0	0	0	0	0	0
11	15001-7	0	0	0	0	0	0	0	0	0	0	0	0
12	15001-8	0	0	0	0	2	0	0	0	0	0	0	0
13	15001-9	0	520	4	0	16	0	256	0	0	52	0	8
14	15001-10	0	0	0	0	50	0	0	0	0	0	0	0
15	15001-11	0	180	32	0	30	0	56	0	0	0	0	0

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- 1 'Received third dose of vaccine intragastrically.
- 2 'Received second, third, and fourth doses of vaccine intragastrically.



TABLE 8. ANTIBODY SECRETING CELL RESPONSES TO C81 BY ELISPOT ASSAY AFTER VACCINATION  
WITH CFA/II ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 14, AND 28  
(E. COLI CVD 15001)

Vaccinee	IgA				IgG				IgM			
	pre	+7	+14	+21	+35	pre	+7	+14	+21	+35	pre	+7
15001-1	0	0	0	0	0	0	0	0	0	0	0	0
15001-2	0	0	0	0	0	0	0	0	0	0	0	0
15001-3 <sup>1</sup>	0	0	0	0	0	0	0	0	0	0	0	0
15001-4	0	0	0	0	0	0	0	0	0	0	0	0
15001-6 <sup>2</sup>	0	0	0	0	0	0	0	0	0	0	0	0
15001-7	0	0	0	0	0	0	0	0	0	0	0	0
15001-8	0	0	0	0	0	0	0	0	0	0	0	0
15001-9	0	128	0	0	12	56	118	0	2	0	0	0
15001-10	0	6	0	0	0	0	0	0	0	0	0	0
15001-11	0	140	0	0	0	0	34	0	0	0	2	0

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- 1 'Received third dose of vaccine intragastrically.
- 2 'Received second, third, and fourth doses of vaccine intragastrically.

TABLE 9. ANTIBODY SECRETING CELL RESPONSES TO C83 BY ELISPOT ASSAY AFTER VACCINATION  
WITH CFA/II ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 14, AND 28  
(E. COLI CVD 15001)

Vaccinee	IgA				IgG				IgM			
	pre	+7	+14	+21	+35	pre	+7	+14	+21	+35	pre	+7
15001-1	0	0	0	0	0	0	0	0	0	0	0	0
15001-2	0	0	0	0	0	0	0	0	0	0	0	0
15001-3'	0	0	0	0	26	0	0	0	0	0	0	0
15001-4	0	0	0	0	98	0	0	0	0	0	0	0
15001-6'	2	0	0	0	0	0	0	0	0	0	0	0
15001-7	0	0	0	0	0	0	0	0	0	0	0	0
15001-8	0	0	0	0	0	0	0	0	0	0	0	0
15001-9	0	580	4	0	6	0	336	0	0	12	0	4
15001-10	0	0	0	0	88	0	0	0	0	0	0	0
15001-11	0	162	32	0	8	0	12	2	0	0	0	0

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- 1 'Received third dose of vaccine intragastrically.
- 2 'Received second, third, and fourth doses of vaccine intragastrically.



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- 1 'Received third dose of vaccine intragastrically.
- 2 'Received second, third, and fourth doses of vaccine intragastrically.

TABLE 11. ANTIBODY SECRETING CELL RESPONSES TO C83 PEPTIDE 792 BY ELISPOT ASSAY AFTER VACCINATION  
WITH CFA/II ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 14, AND 28

(E. COLI CVD 15001)

4	Vaccinee	IgA					IgG					IgM				
		pre	+7	+14	+21	+35	pre	+7	+14	+21	+35	pre	+7	+14	+21	+35
5																
6	15001-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	15001-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	15001-3 <sup>1</sup>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	15001-4	0	0	0	0	2	0	0	0	0	0	0	0	0	0	12
10	15001-6 <sup>2</sup>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	15001-7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6
12	15001-8	2	4	0	0	0	0	0	0	0	0	0	0	0	0	0
13	15001-9	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
14	15001-10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	15001-11	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0

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- 1 'Received third dose of vaccine intragastrically.
- 2 'Received second, third, and fourth doses of vaccine intragastrically.



TABLE 12. JEJUNAL FLUID SECRETORY IGA RESPONSES (RECIPROCAL TITER) TO CFA/II BY ELISA AFTER VACCINATION WITH CFA/II ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 14, AND 28

(E. COLI CVD 15001)

Vaccinee	Pre	+8	+14	+28	+35
15001-1	<4	<4	<4	<4	<4
15001-2	<4	IS	IS	IS	IS
15001-3 <sup>1</sup>	4	<4	NS	4	<4
15001-4	4	<4	4	4	16 <sup>+</sup>
15001-6 <sup>1</sup>	4	NS	NS	NS	8
15001-7	<4	<4	4	4	32 <sup>+</sup>
15001-8	8	4	4	8	8
15001-9	16	≥256	≥256	≥256	256 <sup>+</sup>
15001-10	<4	<4	<4	<4	8 <sup>+</sup>
15001-11	16	32	64	64	32 <sup>+</sup>

<sup>1</sup>Received third dose of vaccine intragastrically.

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1 Received second, third, and fourth doses of vaccine intragastrically.

2 NS indicates no sample. IS indicates inadequate sample.

3 + indicates significant rise in titer.

TABLE 13. SERUM ANTIBODY RESPONSES (RECIPROCAL TITERS) TO CFA/II BY ELISA AFTER VACCINATION  
WITH CFA/II VACCINE ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 21, AND 28  
(E. COLI CVD 15001)

Vaccinee	IgG				IgA				IgM			
	Pre	+7	+28		Pre	+7	+28		Pre	+7	+28	
15001-1	400	100	100		50	50	50		50	50	50	
15001-2	6400	3200	6400		25	<25	<25		50	50	25	
15001-3 <sup>1</sup>	3200	6400	6400		100	200	50		100	50	50	
15001-4	400	200	400		100	400	100 <sup>+</sup>		100	50	50	
15001-6 <sup>2</sup>	1600	1600	1600		200	200	200		25	25	25	
15001-7	400	6400	3200 <sup>+</sup>		400	200	200		25	25	50	
15001-8	3200	400	400		800	800	200		25	25	25	
15001-9	6400	12800	6400		800	3200	3200 <sup>+</sup>		50	50	50	
15001-10	800	400	400		400	400	200		200	400	200	
15001-11	800	1600	1600		400	400	100		25	25	25	

<sup>1</sup>Received third dose of vaccine intragastrically.

<sup>2</sup>Received second, third, and fourth doses of vaccine intragastrically.

+ indicates significant rise in titer.

TABLE 14. SERUM ANTIBODY RESPONSES (RECIPROCAL TITERS) TO CS1 BY ELISA AFTER VACCINATION  
WITH CFA/II VACCINE ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 21, AND 28  
(E. COLI CVD 15001)

Vaccinee	IgG				IgA				IgM			
	Pre	+7	+28		Pre	+7	+28		Pre	+7	+28	
15001-1	<25	<25	<25		25	<25	<25		<25	<25	<25	
15001-2	<25	<25	25		<25	<25	<25		<25	<25	<25	
15001-3 <sup>1</sup>	<25	<25	<25		<25	<25	<25		<25	<25	25	
15001-4	<25	<25	<25		25	<25	<25		25	25	<25	
15001-6 <sup>2</sup>	<25	<25	<25		25	<25	<25		<25	<25	<25	
15001-7	<25	<25	<25		<25	25	<25		<25	<25	<25	
15001-8	<25	<25	<25		25	<25	25		<25	<25	<25	
15001-9	800	800	400		200	200	200		<25	<25	<25	
15001-10	<25	<25	<25		25	<25	<25		25	<25	25	
15001-11	200	3200	800 <sup>+</sup>		100	200	200		<25	<25	<25	

<sup>1</sup>Received third dose of vaccine intragastrically.

<sup>2</sup>Received second, third, and fourth doses of vaccine intragastrically.

+ indicates significant rise in titer.

TABLE 15. SERUM ANTIBODY RESPONSES (RECIPROCAL TITERS) TO CS3 BY ELISA AFTER VACCINATION  
WITH CFA/II VACCINE ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 21, AND 29  
(E. COLI CVD 15001)

Vaccinee	IgG				IgA				IgM			
	Pre	+7	+28		Pre	+7	+28		Pre	+7	+28	
15001-1	50	50	50		50	25	25		<25	25	<25	
15001-2	800	1600	800		<25	<25	<25		25	25	25	
15001-3 <sup>1</sup>	<25	25	50 <sup>+</sup>		50	25	25		25	50	50	
15001-4	25	100	25 <sup>+</sup>		50	50	50		<25	25	25	
15001-6 <sup>2</sup>	200	200	200		200	50	100		25	50	100 <sup>+</sup>	
15001-7	100	50	<25		100	50	25		50	<25	<25	
15001-8	<25	200	100 <sup>+</sup>		25	50	25		<25	50	50 <sup>+</sup>	
15001-9	100	800	800 <sup>+</sup>		50	400	400 <sup>+</sup>		25	50	25	
15001-10	200	100	100		50	25	50		25	25	100 <sup>+</sup>	
15001-11	100	100	200		50	50	50		25	25	<25	

<sup>1</sup>Received third dose of vaccine intragastrically.

<sup>2</sup>Received second, third, and fourth doses of vaccine intragastrically.

+ indicates significant rise in titer.

TABLE 16. CLINICAL AND BACTERIOLOGIC RESPONSES TO CHALLENGE WITH  $5 \times 10^7$  CFU OF  
 ENTEROTOXIGENIC *E. COLI* STRAIN E24377A (0139:H28 LT'ST'CS'CS3') AMONG  
 VACCINEES AND CONTROL VOLUNTEERS (*E. COLI* CVD 15002)

Volunteer	Inoculation Period (hr:min)	Volume of Grade 2, 3 Sample (ml)	No. of Sheds Grade 2, 3	Time (min) (17)	Duration of Fecal Shedding (days)	Pre-Inoculation M (log)
<b>Vaccinees</b>						
15001.1	18:20	1391	10	-	7	$1 \times 10^3$
15001.2	41:30	817	3	-	6	$1 \times 10^8$
15001.3	33:07	1231	9	$1 \times 10^3$ 28	5	$3 \times 10^2$
15001.4	21:18	1012	7	$1 \times 10^3$ 36	7	$1 \times 10^2$
11001.6	-	0	0	-	5	$2 \times 10^2$
15001.7	18:46	6380	19	-	6	$6 \times 10^3$
15001.8	21:19	9132	21	$1 \times 10^3$ 96	5	$5 \times 10^3$
15001.9	-	0	0	-	5	$3 \times 10^2$
15001.10	21:08	1608	14	-	7	$3 \times 10^3$
15001.11	-	0	0	-	5	$2 \times 10^2$
Mean	22:34	2019	10.7	-	5.8	$3 \times 10^3$
<b>Control Volunteers</b>						
15002.1	18:51	1701	0	-	4	$3 \times 10^3$
15002.5	24:01	812	0	-	8	$3 \times 10^3$
15002.6	21:40	177	7	-	4	$2 \times 10^3$
15002.8	12:58	1753	6	$1 \times 10^3$ 41	7	$3 \times 10^3$
15002.9	22:12	1518	11	-	4	$3 \times 10^3$
15002.11	27:14	1253	7	-	7	$4 \times 10^3$
15002.12	20:31	1736	13	-	5	$3 \times 10^3$
15002.13	21:58	740	5	-	6	$5 \times 10^3$
15002.16	48:07	1004	7	-	6	$1 \times 10^3$
15002.21	20:11	1446	18	-	5	$5 \times 10^3$
Mean	22:38	1464	8.6	-	5.8	$3.8 \times 10^3$



TABLE 18. ANTIBODY SECRETING CELL RESPONSES TO C83 PEPTIDES 792 AND 795 BY ELISPOT AFTER CHALLENGE WITH ENTEROTOXIGENIC E. COLI STRAIN E24377A AMONG VACCINEES AND CONTROL VOLUNTEERS  
(E. COLI CVD 15002)

Volunteer	C83 PEPTIDE 792						C83 PEPTIDE 795					
	792		792		792		795		795		795	
	Pre <sup>1</sup>	Post <sup>2</sup>	Pre <sup>1</sup>	Post <sup>2</sup>	Pre <sup>1</sup>	Post <sup>2</sup>	Pre <sup>1</sup>	Post <sup>2</sup>	Pre <sup>1</sup>	Post <sup>2</sup>	Pre <sup>1</sup>	Post <sup>2</sup>
Vaccinees												
15001.1	0	0	0	0	0	0	0	0	0	0	0	0
15001.2	0	0	0	0	0	0	0	0	0	0	0	0
15001.3 <sup>3</sup>	0	0	0	0	0	0	0	0	0	0	0	0
15001.4	0	0	0	0	0	0	0	0	0	0	0	0
15001.5 <sup>4</sup>	0	0	0	0	0	0	0	0	0	0	0	0
15001.6	0	0	0	0	0	0	0	0	0	0	0	0
15001.7	0	0	0	0	0	0	0	0	0	0	0	0
15001.8	22	2	0	0	0	0	0	0	0	0	0	0
15001.9 <sup>5</sup>	0	0	0	0	0	0	0	0	0	0	0	0
15001.10	7	0	0	0	0	0	0	0	0	0	0	0
15001.11 <sup>6</sup>	0	0	0	0	0	0	0	0	0	0	0	0
Controls												
15002.1	0	0	0	0	0	0	0	0	0	0	0	0
15002.5	0	0	0	0	0	0	0	0	0	0	0	0
15002.6	0	0	0	0	0	0	0	0	0	0	0	0
15002.8	0	0	0	0	0	0	0	0	0	0	0	0
15002.9	0	0	0	0	0	0	0	0	0	0	0	0
15002.11	0	0	0	0	0	0	0	0	0	0	0	0
15002.12	0	0	0	0	0	0	0	0	0	0	0	0
15002.13	0	0	0	0	0	0	0	0	0	0	0	0
15002.16	2	0	0	0	0	0	0	0	0	0	0	0
15002.21	0	0	0	0	0	0	0	0	0	0	0	0

<sup>1</sup>Prechallenge cell count of vaccinee biologically  
<sup>2</sup>Post second, third, and fourth doses of vaccine biologically  
<sup>3</sup>Pre<sup>1</sup> indicates before challenge, for vaccinees; Pre<sup>1</sup> is day 37 after first dose of vaccine  
<sup>4</sup>Vaccinees who did not become ill



TABLE 19. ANTIBODY SECRETING CELL RESPONSES TO 0139 LIPOPOLYSACCHARIDE (LPS) AND HEAT LABILE ENTEROTOXIN (LT) BY ELISPOT AFTER CHALLENGE WITH ENTEROTOXIGENIC E. COLI STRAIN E24377A AMONG VACCINEES AND CONTROL VOLUNTEERS (E. COLI CVD 15002)

Volunteer	0139 LPS										LT									
	Day 1		Day 2		Day 3		Day 4		Day 5		Day 1		Day 2		Day 3		Day 4		Day 5	
	Pos <sup>1</sup>	Pos <sup>2</sup>	Pos <sup>1</sup>	Pos <sup>2</sup>	Pos <sup>1</sup>	Pos <sup>2</sup>	Pos <sup>1</sup>	Pos <sup>2</sup>	Pos <sup>1</sup>	Pos <sup>2</sup>	Pos <sup>1</sup>	Pos <sup>2</sup>	Pos <sup>1</sup>	Pos <sup>2</sup>	Pos <sup>1</sup>	Pos <sup>2</sup>	Pos <sup>1</sup>	Pos <sup>2</sup>	Pos <sup>1</sup>	Pos <sup>2</sup>
Vaccinees																				
15001.1	10	116	0	112	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001.3 <sup>1</sup>	0	200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001.4	0	500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001.5 <sup>2,4</sup>	0	190	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001.7	0	314	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001.8	24	240	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001.9 <sup>4</sup>	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001.10	2	200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001.11 <sup>4</sup>	0	80	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Controls																				
15002.1	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
15002.2	0	400	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
15002.3	0	117	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002.4	0	317	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
15002.5	0	440	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15002.6	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
15002.7	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
15002.8	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
15002.9	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
15002.10	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
15002.11	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
15002.12	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
15002.13	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
15002.14	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
15002.15	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100

<sup>1</sup> Positives only at various concentrations.  
<sup>2</sup> Positives only at various concentrations.  
<sup>3</sup> Positives only at various concentrations.  
<sup>4</sup> Positives only at various concentrations.

1                   TABLE 20. IMMUNE RESPONSES AS MEASURED BY ANTIBODY SECRETING CELLS (ASC) AND BY JEJUNAL  
2                   FLUID SECRETORY IGA AFTER VACCINATION WITH CFA/II ENCAPSULATED IN BIODEGRADABLE  
3                   MICROSPHERES ON DAYS 0, 7, 14, AND 28

Immunologic Assay	Number of Responders <sup>1</sup>	Geometric mean peak number of spots per 10 <sup>6</sup> PBMC (ASC) or reciprocal antibody titer (sIgA)
ASC IgA anti-CFA/II	5/10	44
ASC IgA anti-CS1	3/10	48
ASC IgA anti-CS3	5/10	116
Jejunal fluid sIgA anti-CFA/II	5/10	42

<sup>1</sup>Responses that had occurred by day 35 after the first dose of vaccine, i.e., day 7 after the fourth dose

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TABLE 21. IMMUNE RESPONSES AFTER WILD-TYPE ETEC CHALLENGE AS MEASURED BY  
ANTIBODY SECRETING CELLS (ASC) AND BY JEJUNAL FLUID SECRETORY IGA IN  
UNIMMUNIZED CONTROL VOLUNTEERS

Immunologic Assay	Number of Responders <sup>1</sup>	Geometric mean peak number of spots per 10 <sup>6</sup> PBMC (ASC) or reciprocal antibody titer (sIgA)
ASC IgA anti-CFA/II	9/10	88
ASC IgA anti-CS1	4/10	58
ASC IgA anti-CS3	9/10	161
Jejunal fluid sIgA anti-CFA/II	6/9	72

<sup>1</sup>Measured day 7 after challenge

TABLE 22. PRE-CHALLENGE IMMUNITY AND CLINICAL AND BACTERIOLOGIC RESPONSE  
TO CHALLENGE WITH  $5 \times 10^8$  CFU OF ENTEROTOXIGENIC E. COLI STRAIN E24377A  
(0139:H28 LT<sup>+</sup>ST<sup>+</sup>CS1<sup>+</sup>CS3<sup>+</sup>) AMONG VACCINEES AND CONTROL VOLUNTEERS

	Vaccinees	Controls
Number with $> 4$ IgA anti-colonization factor ASC <sup>1</sup> per $10^6$ PBMC on the day of challenge <sup>2</sup>	8/10	4/10
Geometric mean number of IgA anti-colonization factor ASC per $10^6$ PBMC on the day of challenge <sup>3</sup>	25	14
Attack Rate for Diarrhea	7/10	10/10
Volume of Diarrhea	2819 ml	1464 ml
Peak Stool Excretion of Challenge Organism	$3 \times 10^8$ cfu	$4 \times 10^8$ cfu

<sup>1</sup>Including anti-CFA/II, anti-CS1, and/or anti-CS3

<sup>2</sup>Day 57 after the first dose of vaccine

<sup>3</sup>Among those with  $> 4$  IgA ASC before challenge (n=8 for vaccinees and n=4 for controls)

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## DISCUSSION

The potential advantage of microcapsules lies in their ability to be programmed during fabrication into forms that have quite difference release profiles, including slow and steady release, multiple bursts of antigen over a period of time, or combinations of release forms. Sieving allows choice of microcapsule size, and the ability of DL-PLG to sequester antigen from the host's immune system until release occurs enhances control over exposure of the recipient's immune system to antigen over a sustained period of time. These characteristics provided the impetus for these studies as they indicate potential for achieving the effects of a multiple injection regimen by controlling release in vivo after a single injection.

The results of these studies are important for gaining an under standing of the fundamental differences between the manner in which alum and microcapsules interact with the immune system. The antigen release studies showed that alum firmly bound the antigen on its surface, whereas the microcapsules sequestered the antigen load within the interstices of an immunologically inert polymer. Release of antigen from microcapsules was spontaneous and gradual while antigen release from alum wa probably enzymatically mediated within host macrophages. Alum thus performed at least two useful functions as an adjuvant: by bearing its entire load of antigen upon its surface, it provided a large single exposure of antigen to the host; and, by being

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1 readily phagocytized by host macrophages, it served as a means  
2 of targeting the antigen to the immune system.

3 In order for microcapsules to be efficacious as a vaccine .  
4 delivery system, a means of incorporating the two properties  
5 common to alum adjuvant must be devised. These properties, which  
6 where discussed above, are targeting antigen to the immune  
7 system and delivering the antigen load in a single concentrated  
8 pulse at its target. A gradual, sustained release of free  
9 antigen, as was achieved with the 100 micron microcapsules used  
10 in these studies, could be expected to elicit an immune response  
11 similar to that seen with either regimen b or regimen c (Table  
12 5), where multiple injections of small doses were employed. In  
13 fact, as shown in Table 3, the microencapsulated immunogen  
14 elicited a response similar to that achieved with regimen b.  
15 This is probably due to the fact that the microcapsules release  
16 approximately 10% of their antigenic load immediately after  
17 injection.

18 Microcapsules with extended release patterns tend to be  
19 large (>10 microns in diameter) and thus fail to be readily  
20 phagocytized. In order for the larger microcapsules with  
21 prolonged antigen release characteristics to be efficacious, the  
22 antigen eventually released from those microcapsules would have  
23 be in a form which targeted and concentrated it within the  
24 recipient's immune system. This might be effectively achieved by  
25 microencapsulation of antigen coated alum or by

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1 microencapsulating clusters of smaller (<10 microns)  
2 microcapsules.

3 Microcapsules under 10 microns in diameter tend to be  
4 readily phagocytized and also tend to under go rapid spontaneous  
5 degradation due to their high surface to volume ratio. These  
6 smaller microcapsules would be well suited for eliciting a  
7 primary response if their pulse of antigen release could be  
8 programmed to occur after phagocytosis.

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1           We Claim:

2           1. An immunostimulating composition comprising  
3           encapsulating- microspheres, which may contain a  
4           pharmaceutically-acceptable adjuvant, wherein said microspheres  
5           having a diameter between 1 nanogram (ng) to 10 microns (um) are  
6           comprised of (a) a biodegradable-biocompatible poly (DL-lactide-  
7           co-glycolide) as the bulk matrix, wherein the relative ratio  
8           between the amount of lactide and glycolide components are  
9           within the range of 40:60 to 0:100 and (b) an immunogenic  
10          substance comprising Colony Factor Antigen (CFA/II), hepatitis B  
11          surface antigen (HBsAg), or a physiologically similar antigen  
12          that serves to elicit the production of antibodies in animal  
13          subjects.

14          2. An immunostimulating composition according to Claim 1  
15          wherein the amount of said immunogenic substance is within the  
16          range of 0.1 to 1.5% based on the volume of said bulk matrix.

17          3. An immunostimulating composition according to Claim 2  
18          wherein the relative ratio between the lactide and glycolide  
19          component is within the range of 48:52 to 58:42.

20          4. An immunostimulating composition according to Claim 2  
21          wherein the size of more than 50% of said microspheres is  
22          between 5 to 10 um in diameter by volume.

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1           5. An immunostimulating composition according to Claim 1  
2       wherein the immunogenic substance is the synthetic peptide  
3       representing the peptide fragment beginning with the amino acid  
4       residue 63 through 78 of Pilus Protein CS3, said residue having  
5       the amino acid sequence, 63(Ser-Lys-Asn-Gly-Thr-Val-Thr-Try-Ala-  
6       His-Glu-Thr-Asn-Asn-Ser-Ala).

7           6. A vaccine comprising an immunostimulating composition  
8       of Claim 4 and a sterile, pharmaceutically-acceptable carrier  
9       therefor.

10          7. A vaccine comprising an immunostimulating composition  
11       of Claim 6 wherein said immunogenic substance is Colony Factor  
12       Antigen (CFA/II).

13          8. A vaccine comprising an immunostimulating composition  
14       of Claim 6 wherein said immunogenic substance is hepatitis B  
15       surface antigen (HBsAg).

16          9. A method for the vaccination against bacterial  
17       infection comprising administering to a human, an  
18       antibactericidally effective amount of a composition of Claim 6.

19          10. A method according to Claim 8 wherein the bacterial  
20       infection is caused by a bacteria selected from the group  
21       consisting essentially of Salmonella typhi, Shigella Sonnei,

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1     Shigella Flexneri, Shigella dysenteriae, Shigella boydii,  
2     Escheria coli, Vibrio cholera, versinia, staphylococcus,  
3     clostridium, and campylobacter.

4             11. A method for the vaccination against viral infection  
5     comprising administering to a human an antivirally effective  
6     amount of a composition of Claim 8.

7             12. A diagnostic assay for bacterial infections comprising  
8     a composition of Claim 4.

9             13. A method of preparing an immunotherapeutic agent  
10    against infections caused by a bacteria comprising the step of  
11    immunizing a plasma donor with a vaccine according to Claim 7  
12    such that a hyperimmune globulin is produced which contains  
13    antibodies directed against the bacteria.

14            14. A method preparing an immunotherapeutic agent against  
15    infections caused by a virus comprising the step of immunizing a  
16    plasma donor with a vaccine according to Claim 8 such that  
17    hyperimmune globulin is produced which contains antibodies  
18    directed against the hepatitis B virus.

19            15. An immunotherapy method comprising the step of  
20    administering to a subject an immunostimulatory amount of  
21    hyperimmune globulin prepared according to Claim 13.

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1           16. An immunotherapy method comprising the step of  
2           administering to a subject an immunostimulatory amount of  
3           hyperimmune globulin prepared according to Claim 14.

4           17. A method for the protection against infection of a  
5           subject by enteropathogenic organisms or hepatitis B virus  
6           comprising administering to said subject an immunogenic amount  
7           of an immunostimulating composition of Claim 3.

8           18. A method according to Claim 17 wherein the  
9           immunostimulating composition is administered orally.

10          19. A method according to Claim 17 wherein the  
11          immunostimulating composition is administered parenterally.

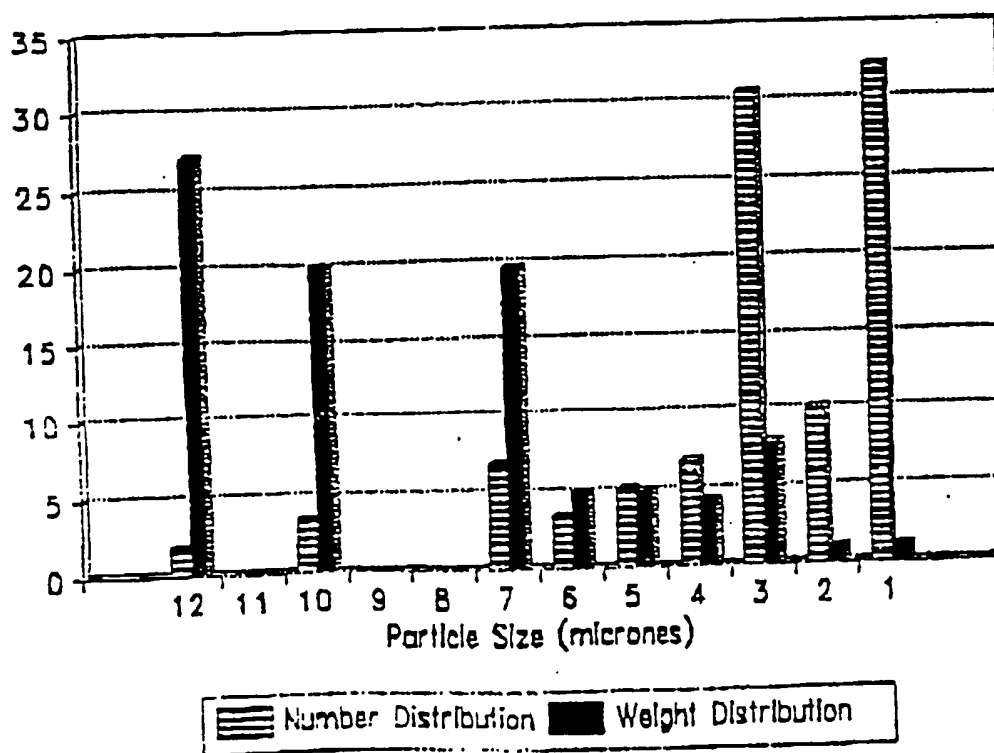
12          20. A method according to Claim 17, wherein the  
13          immunostimulating composition is administered in four separate  
14          doses on day 0, day 7, day 14, and day 28.

15          21. A method according to Claim 17 wherein the immunogenic  
16          substance is the synthetic peptide representing the peptide  
17          fragment beginning with the amino acid residue 63 through 78 of  
18          Pilus Protein CS3 said residue having the amino acid sequence  
19          63(Ser-Lys-Asn-Gly-Thr-Val-Thr-Try-Ala-His-Glu-Thr-Asn-Asn-Ser-  
20          Ala).

Figure 1

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## Particle Size Distribution



# Figure 2

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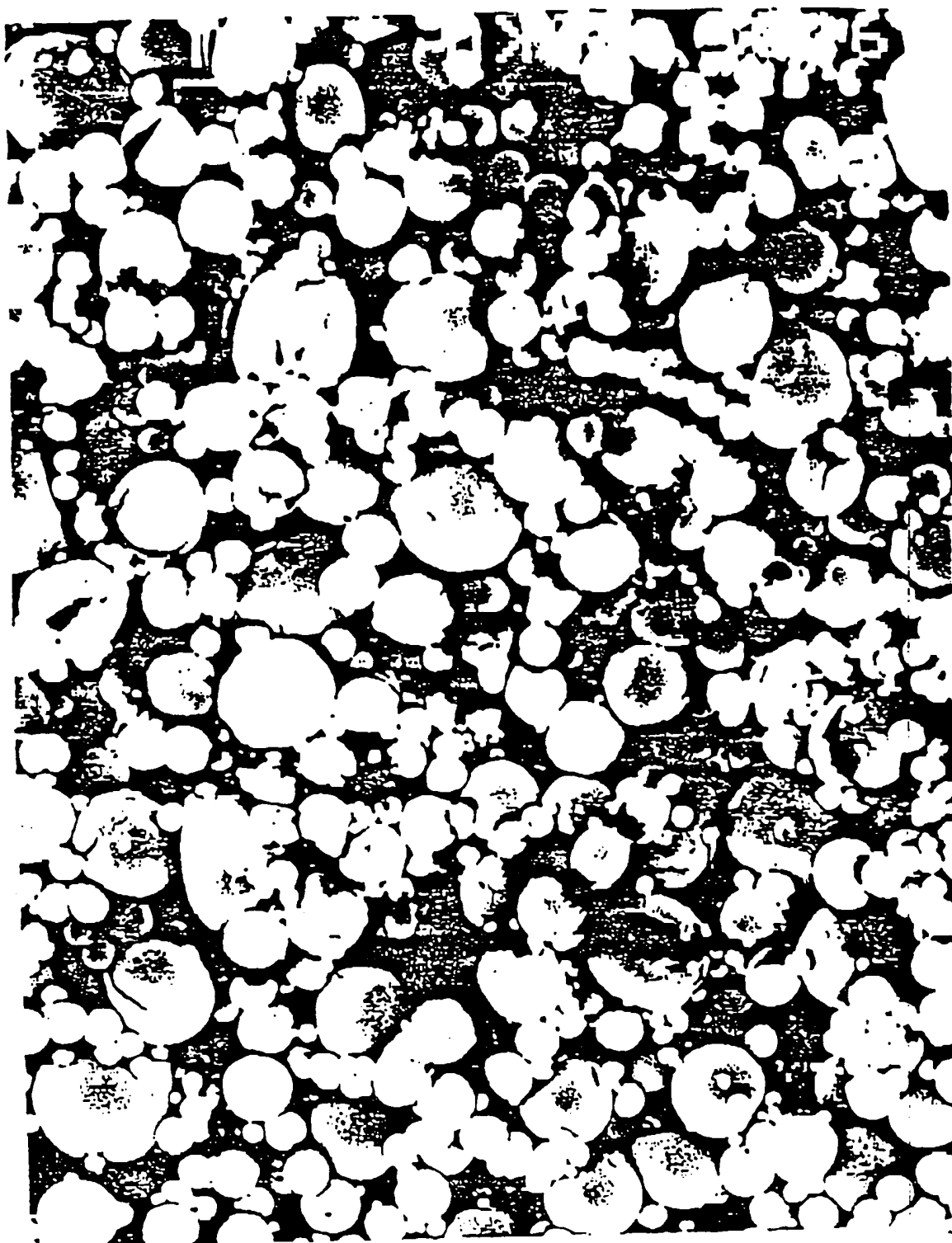


Fig. 3(a)  
Spleen

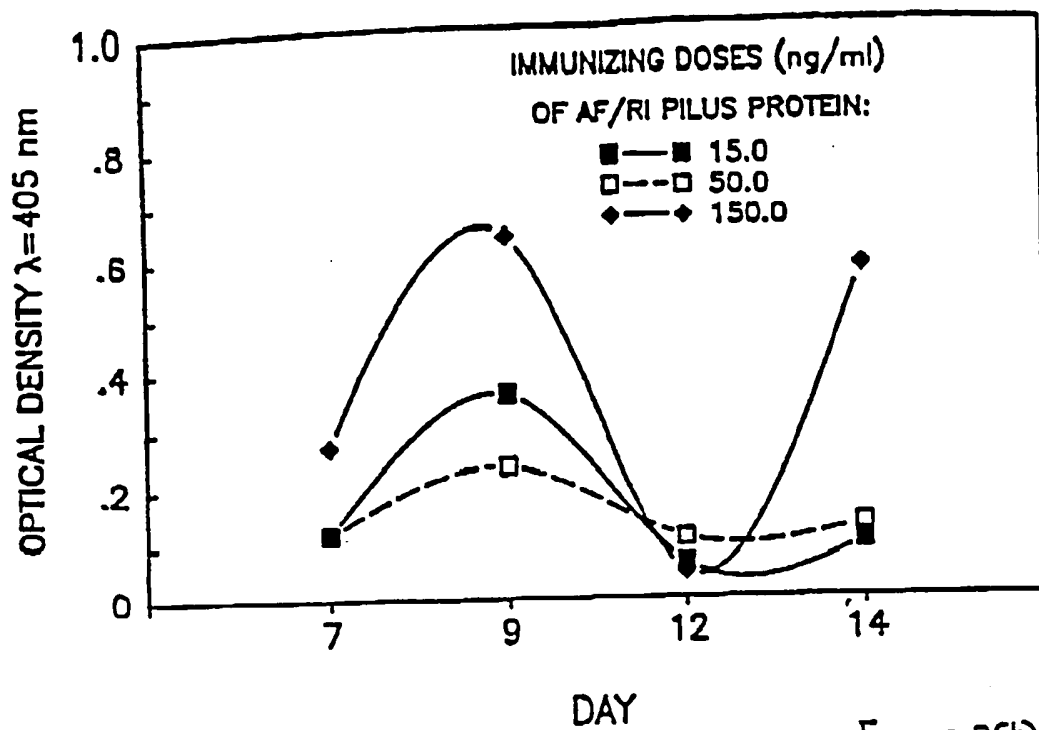
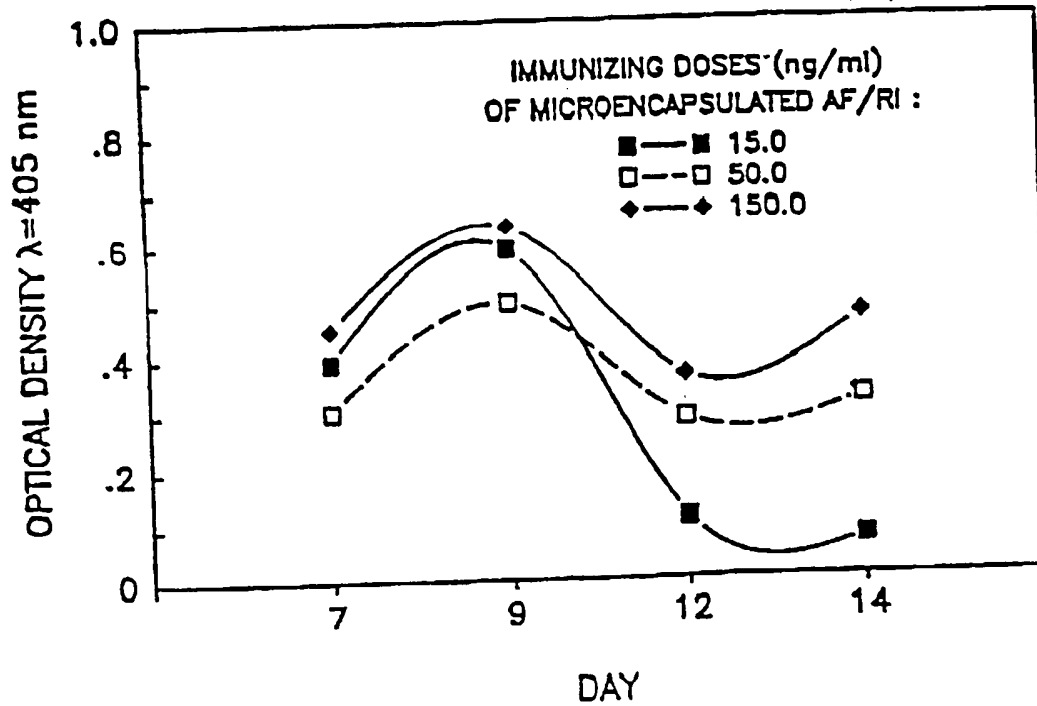


Figure 3(b)



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Figure 4(a)

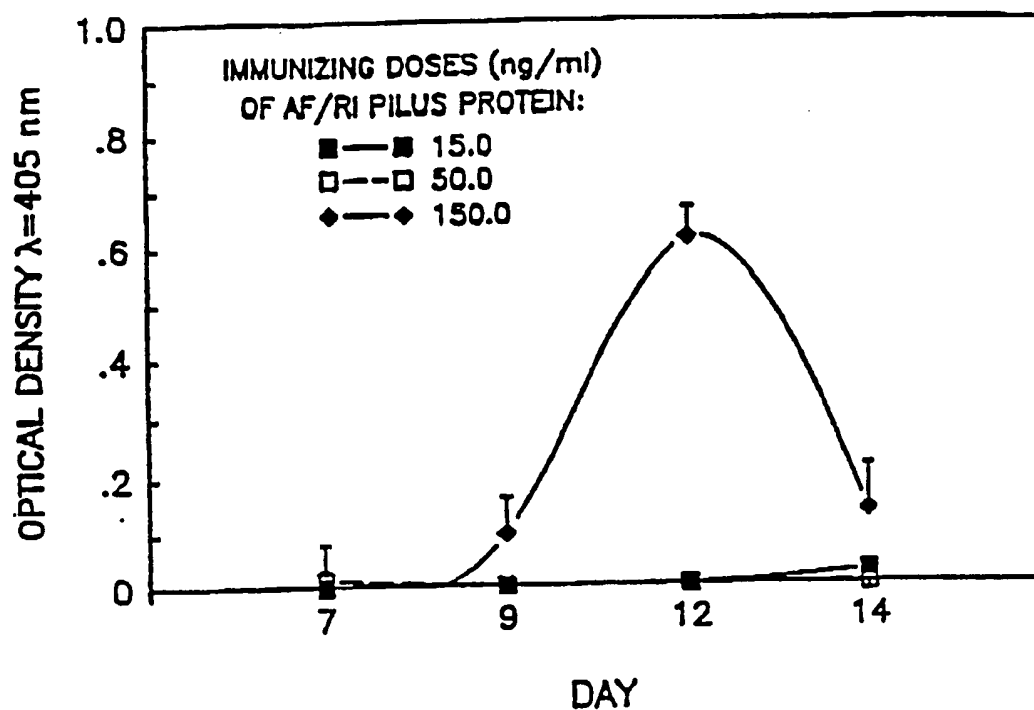
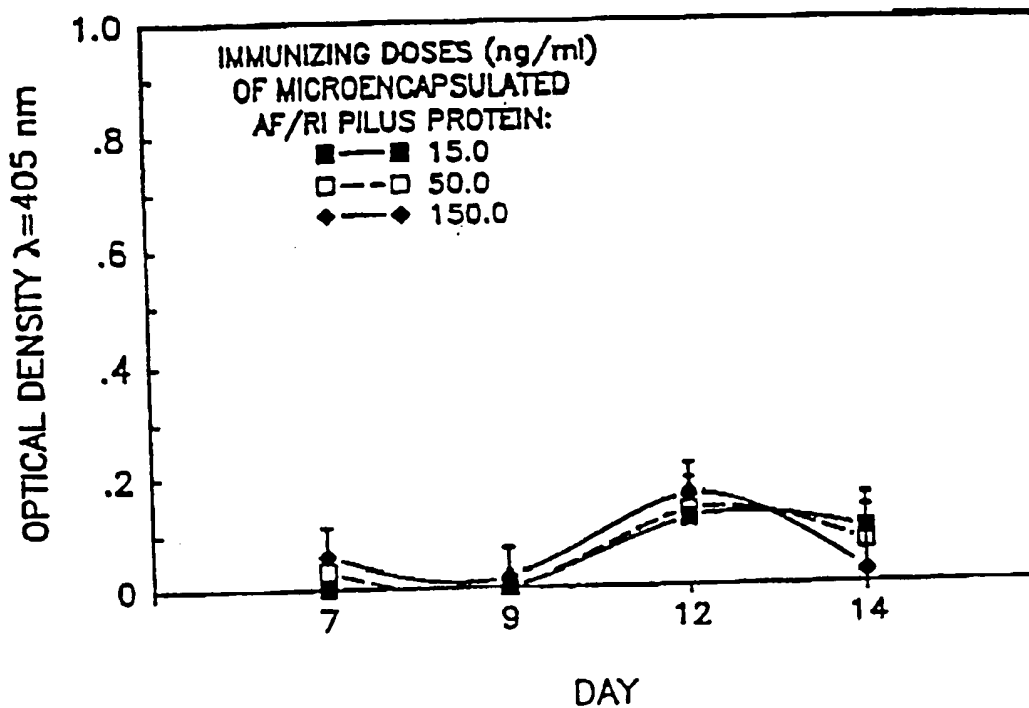


Figure 4(b)





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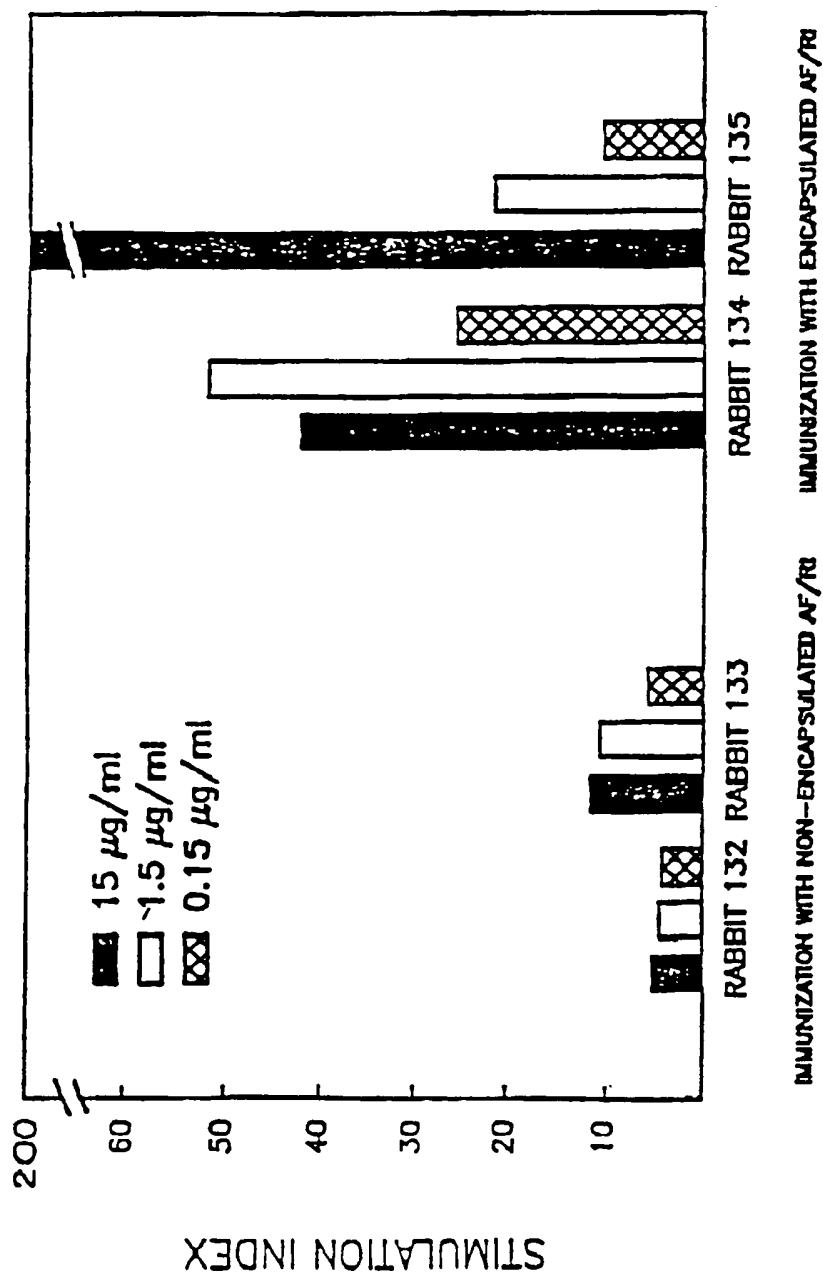
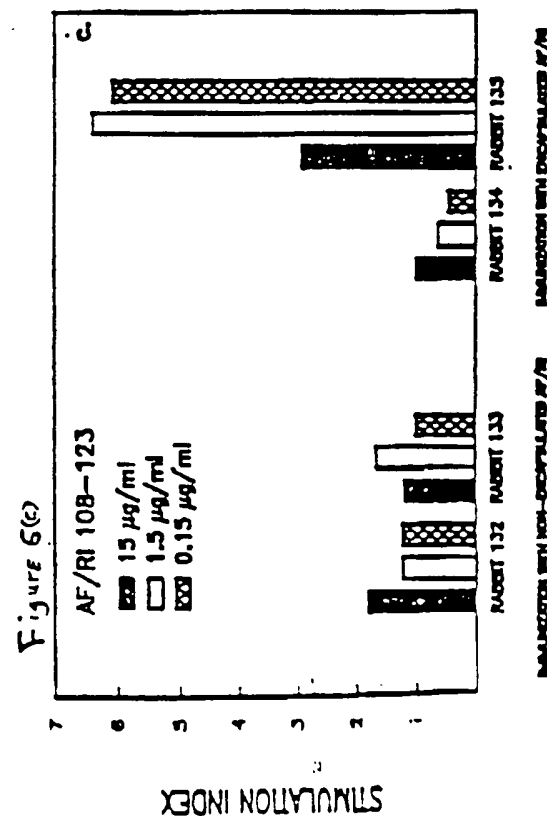
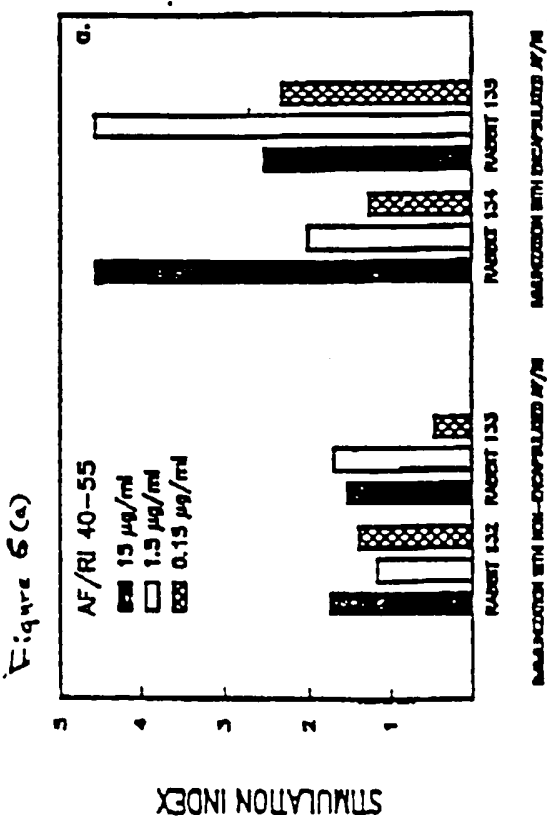
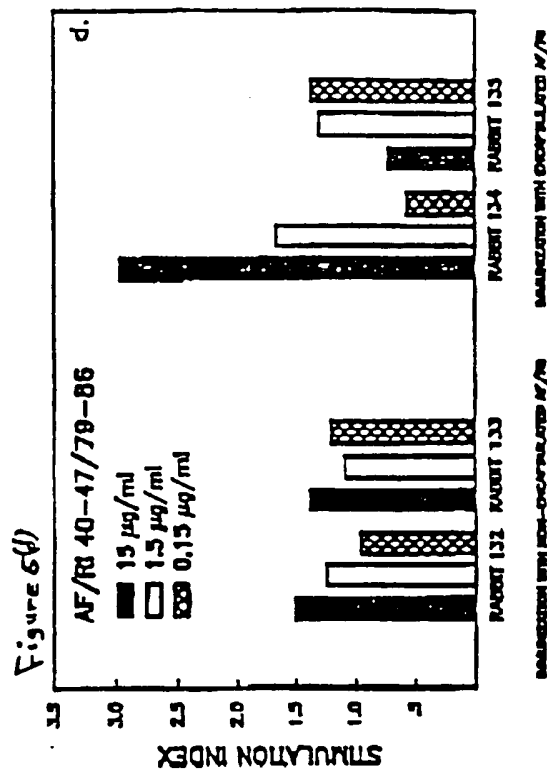
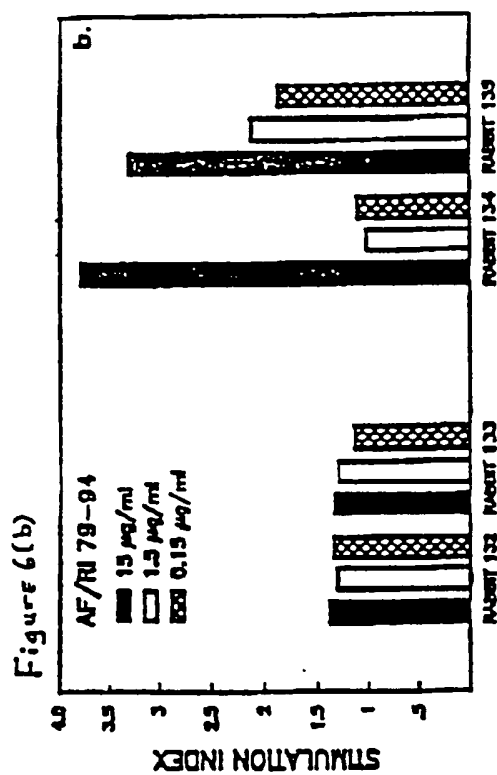


Figure 5



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Fig. 7  
Peyer's patch

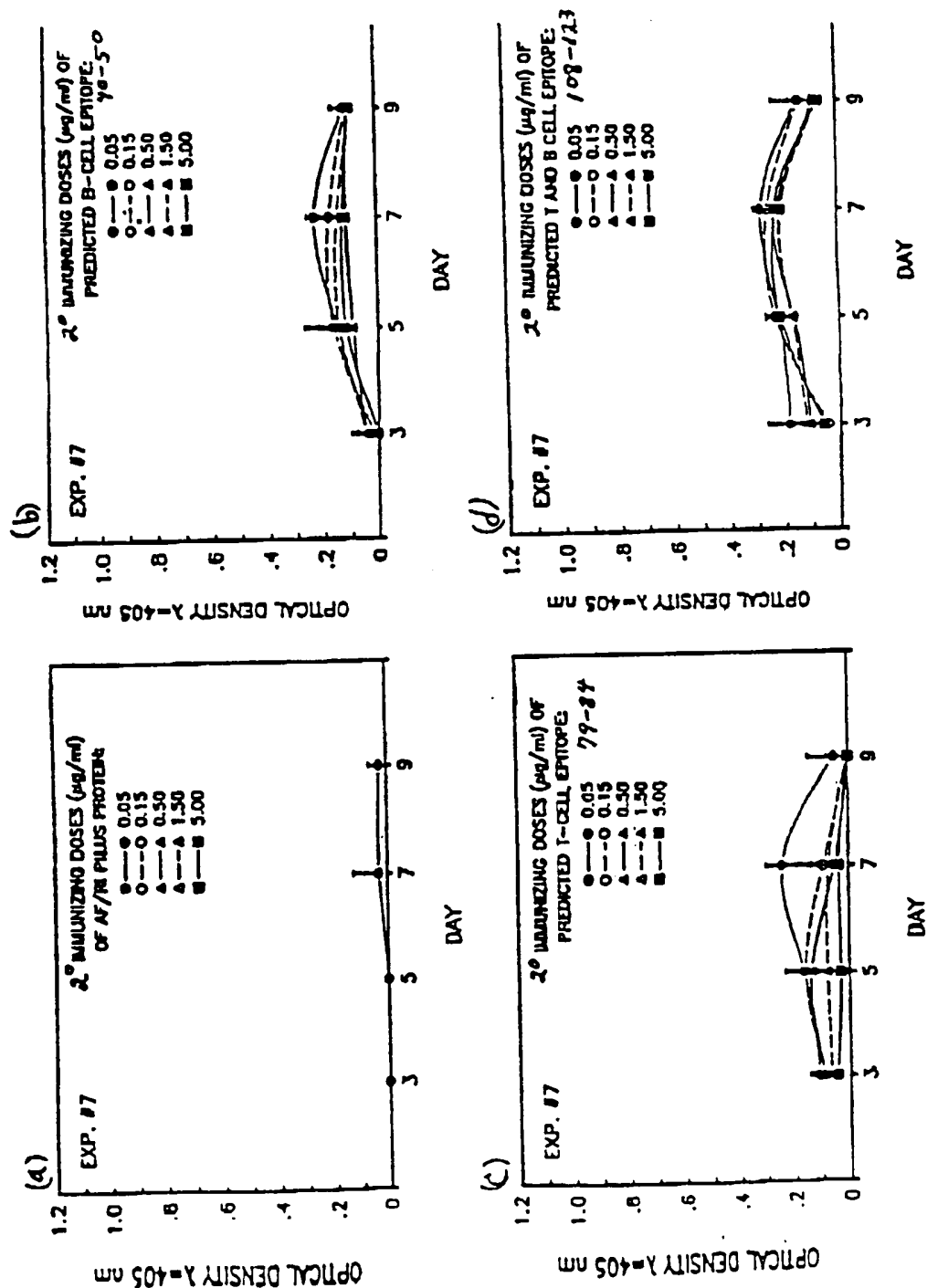
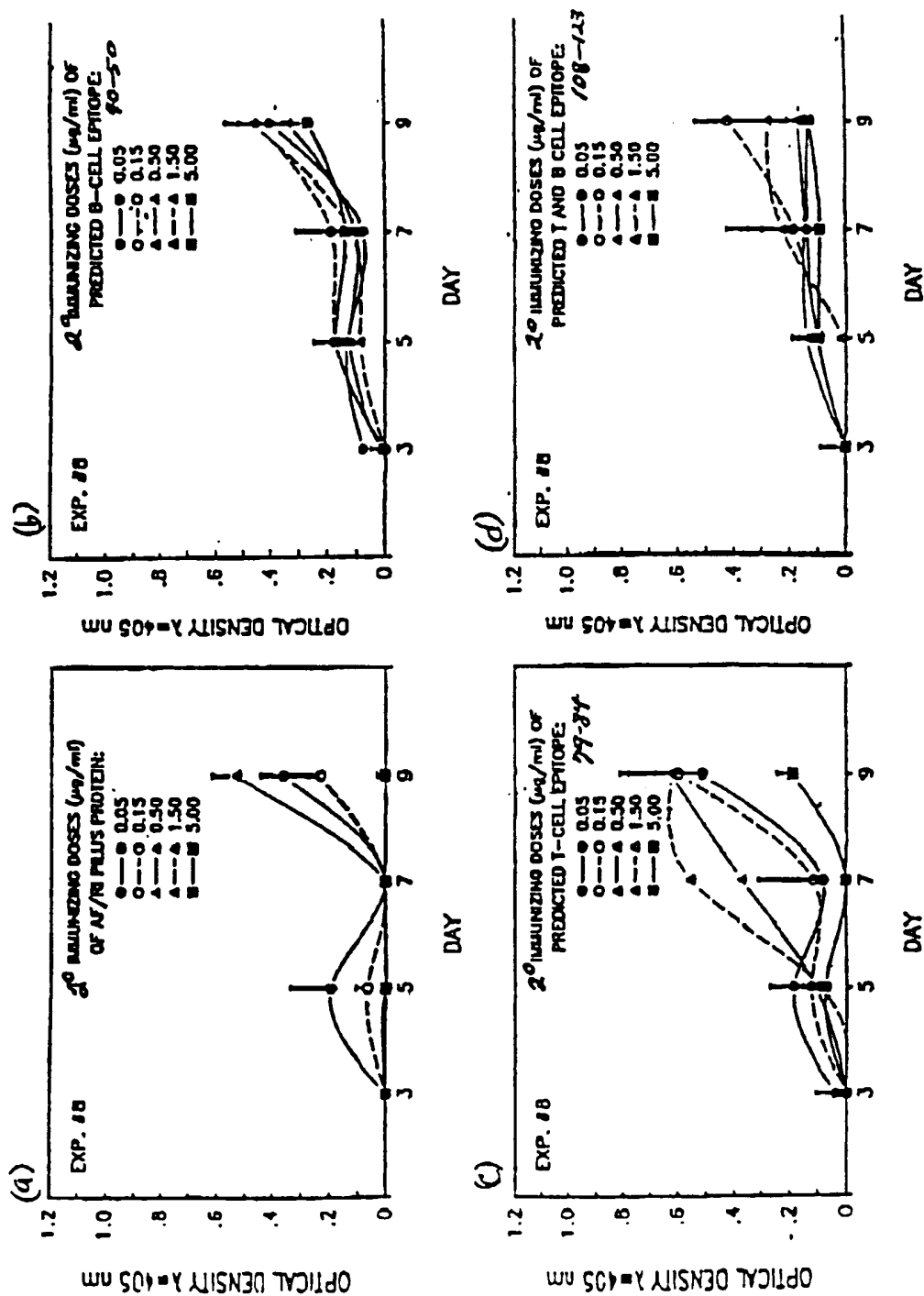
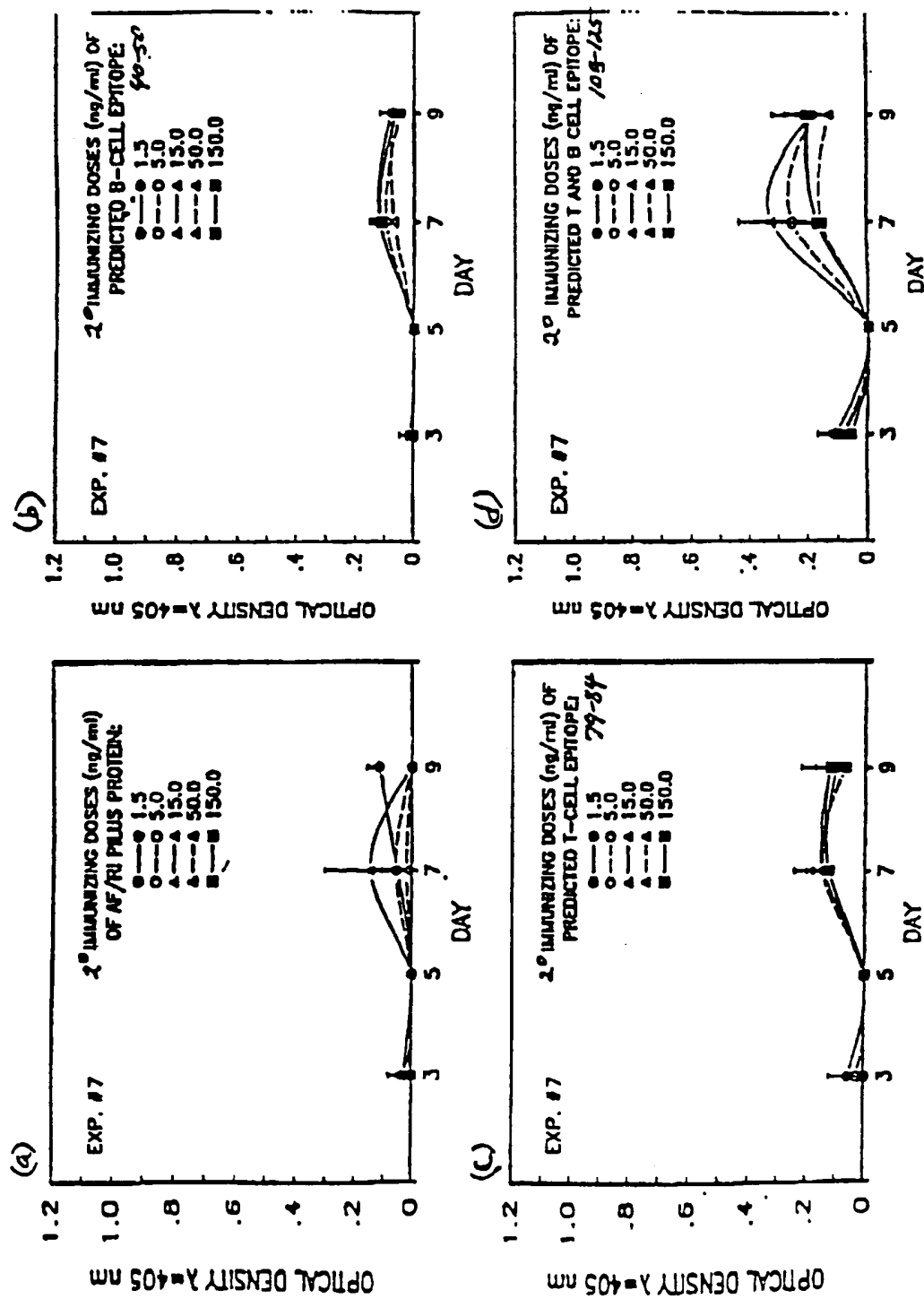


Fig. 8



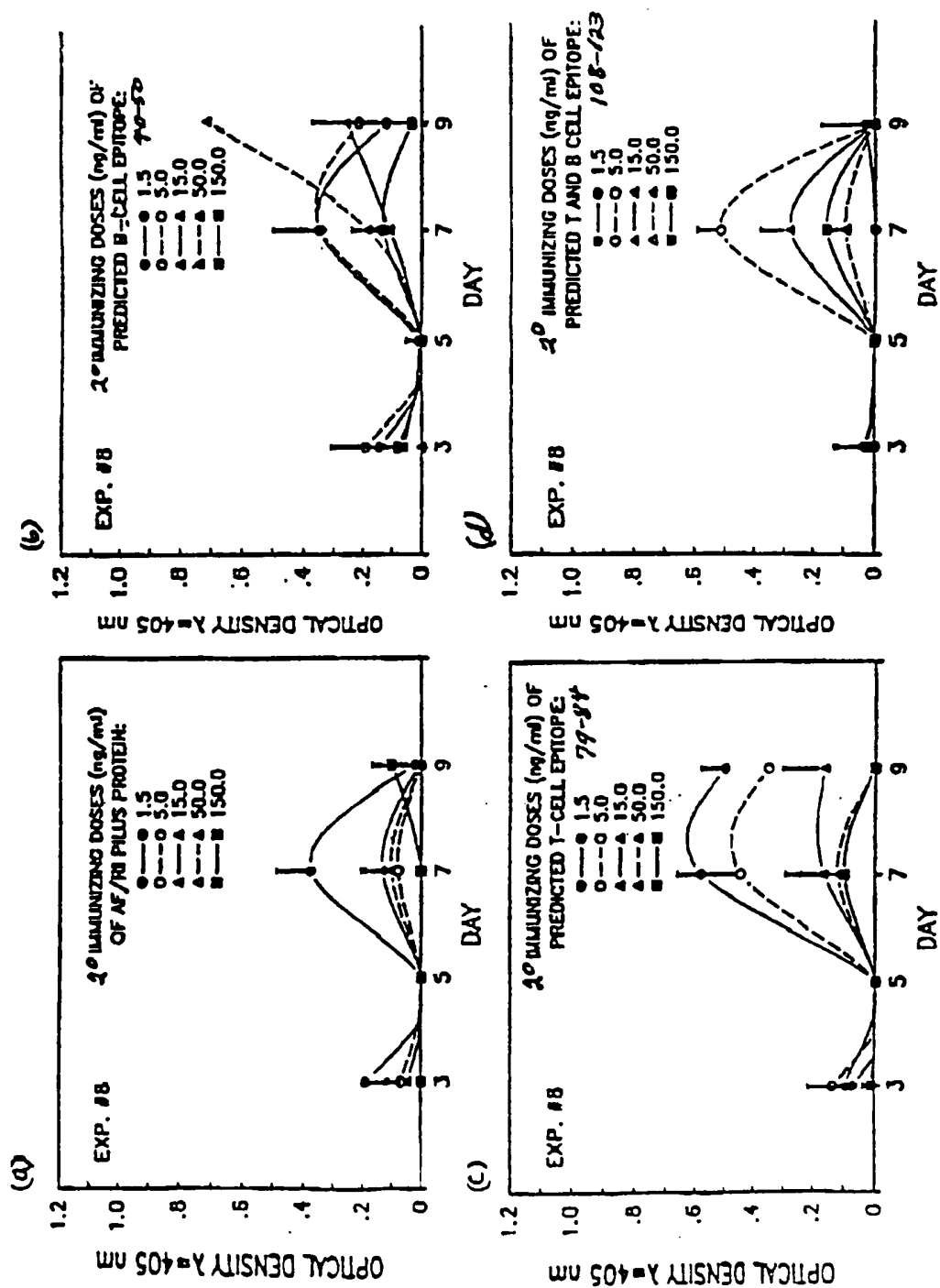
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Fig. 9



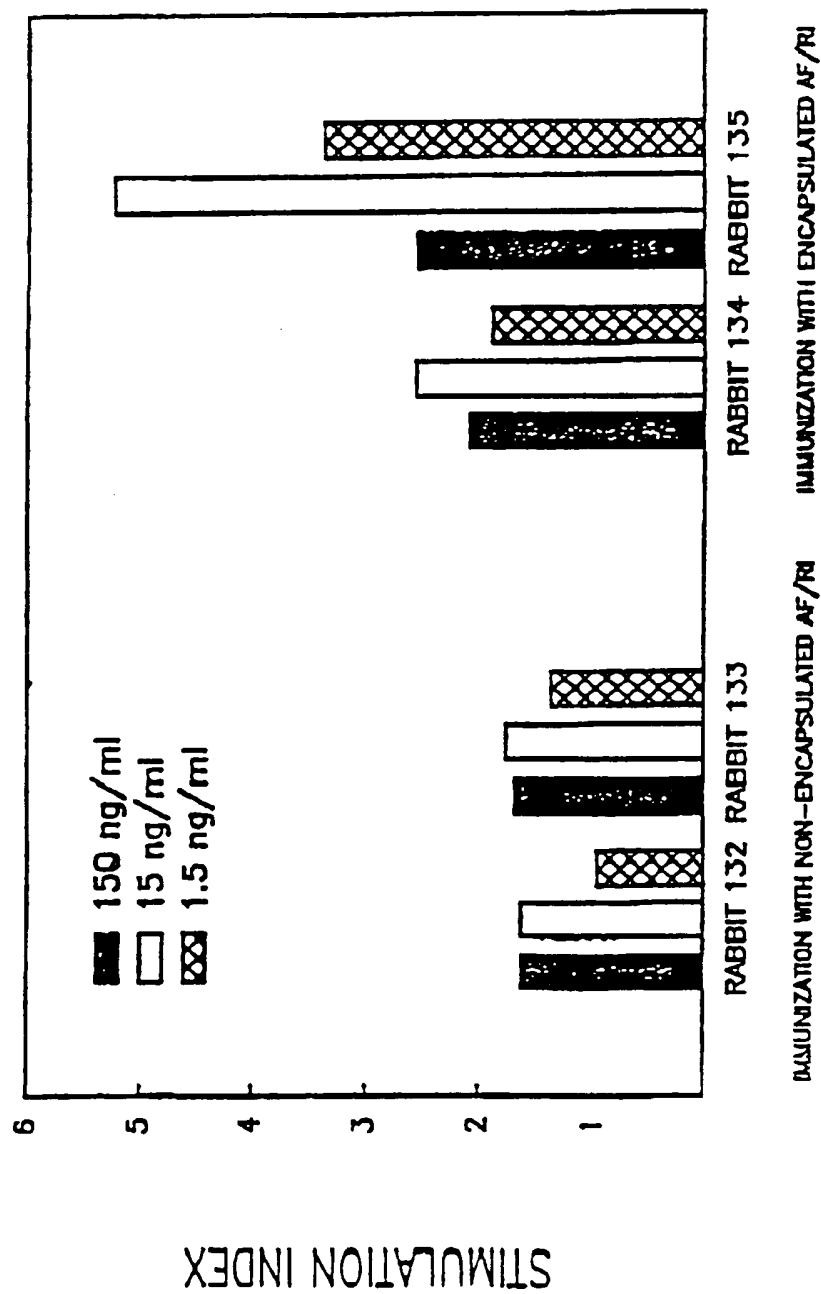
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Fig. 80



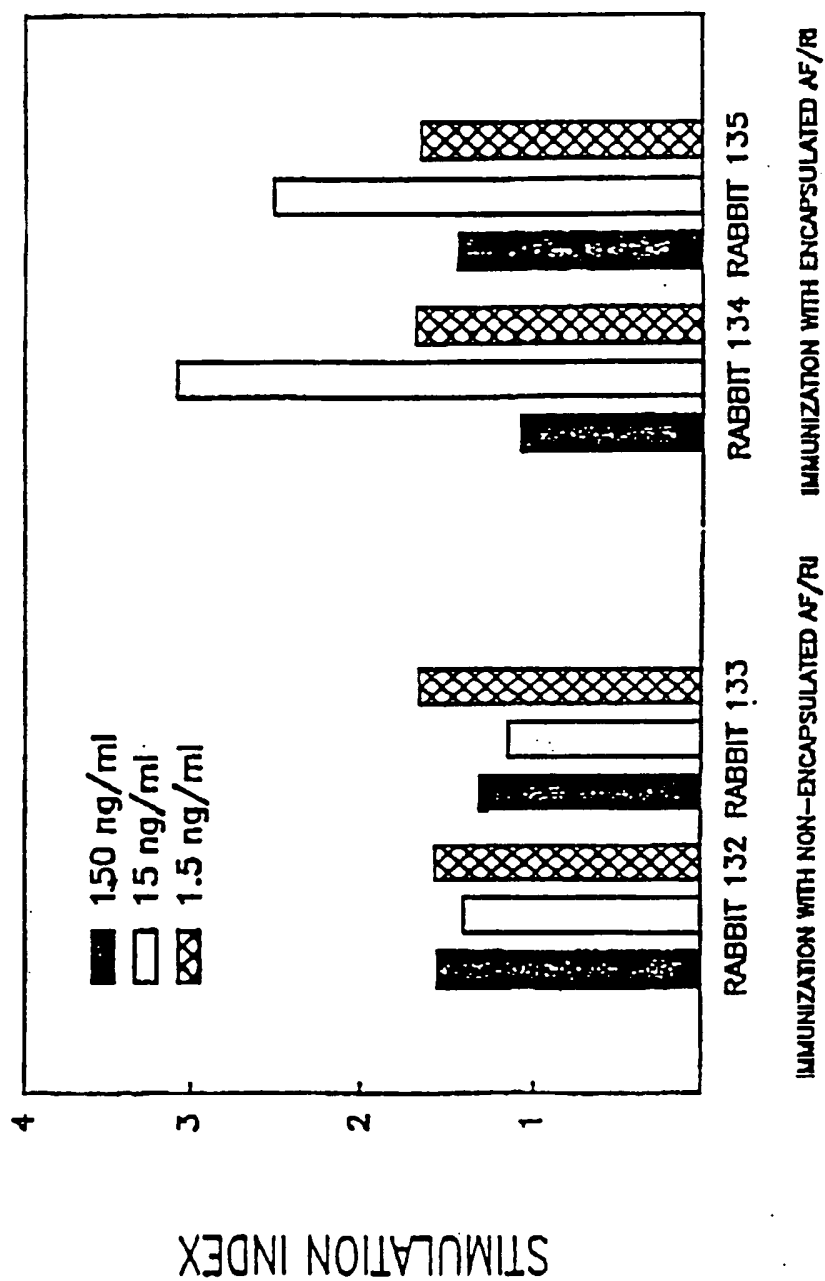
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Fig. 10



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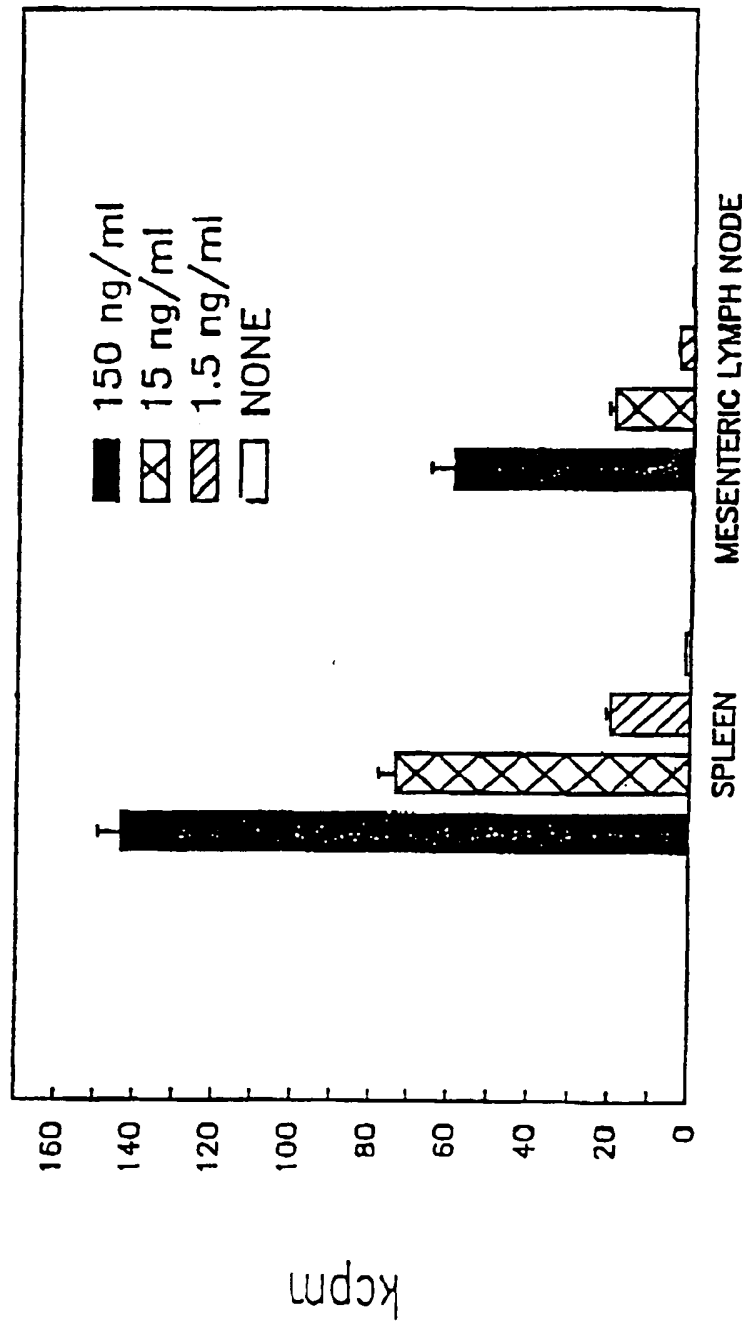
Fig. 12





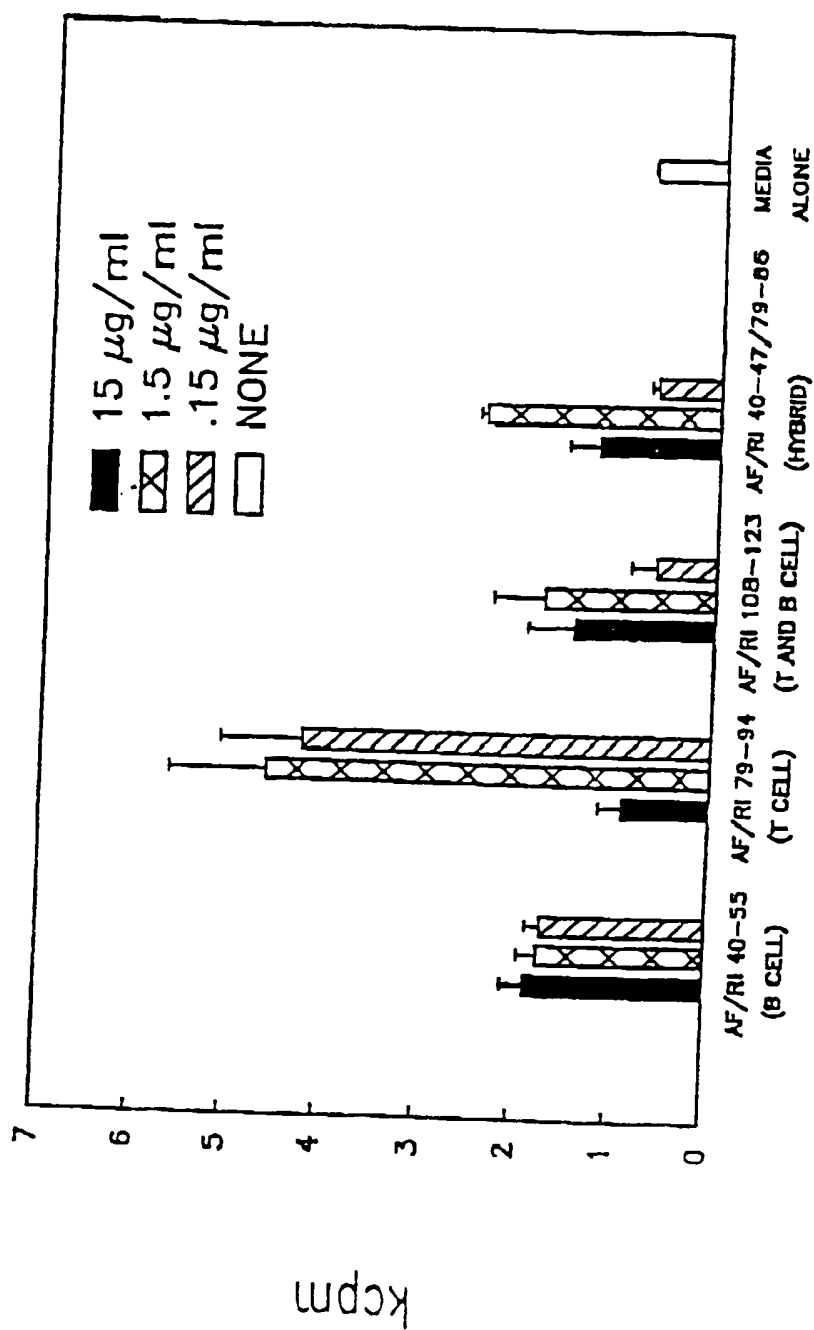
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Fig 13



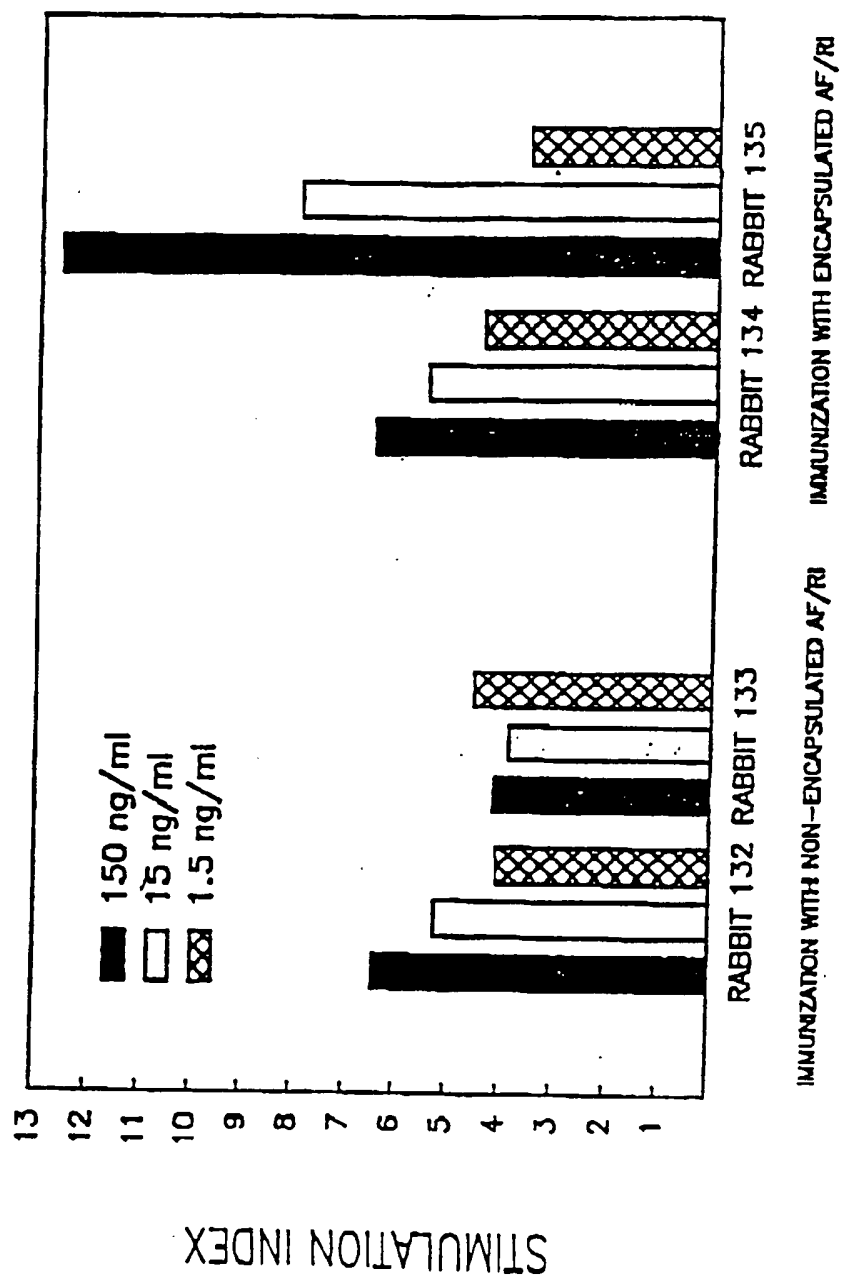
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Fig. 14

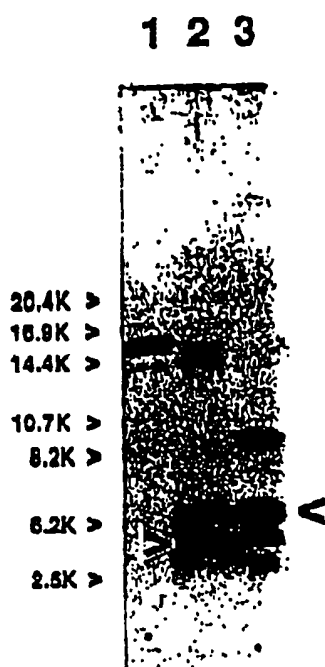


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Fig. 15



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**A****B**

Lane 2	LADTPQLTDVLN <u>E</u> TVQMP	(52-79)
Lane 3	SYRVMTQVETN <u>D</u> ATKKVIV	(42-60)

Figure 16

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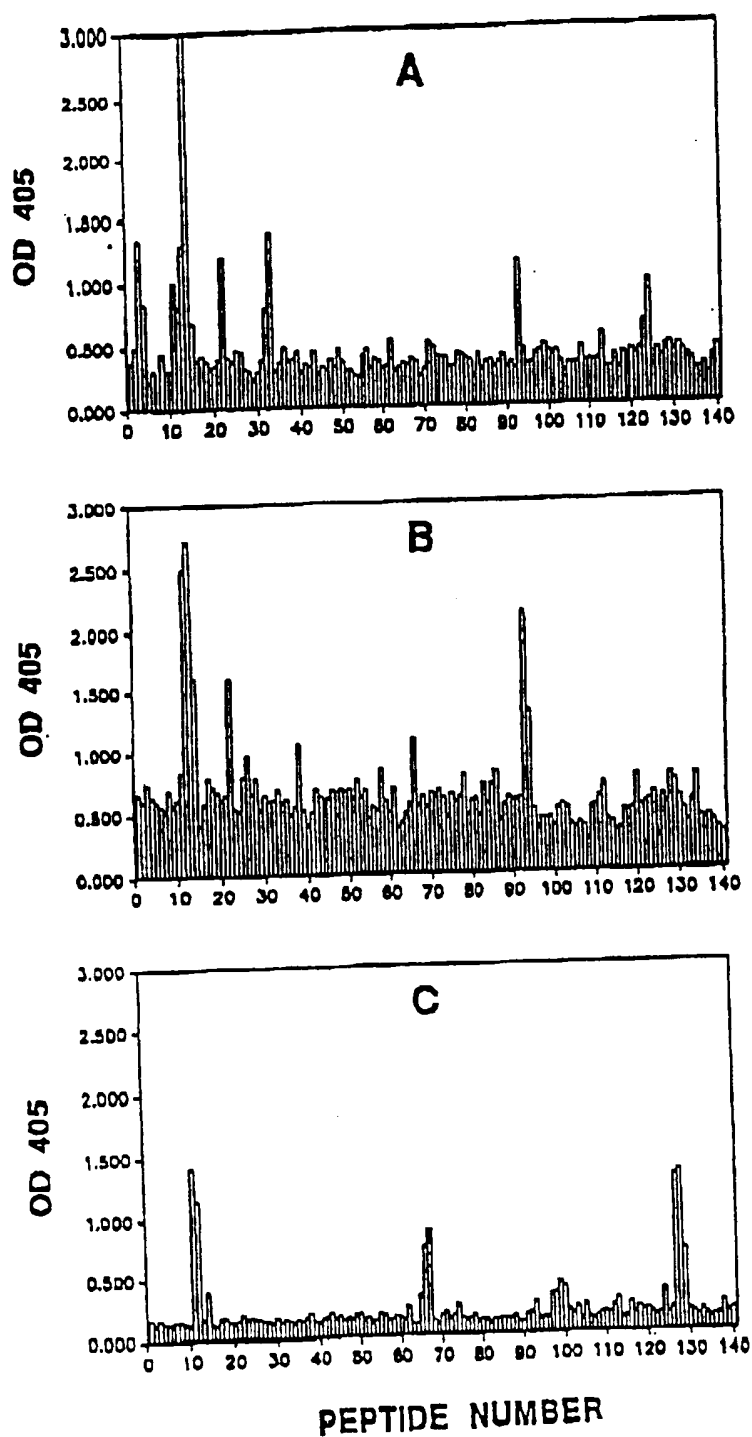


Figure 17

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282 V E K N I T V T A S V D P P V I D L L Q A D P G N A L P S A V K L A Y S P A S K T F E Z E Y R V M T Q V H 50  
184D V E K N I T V T A S V D P P V I D L L Q A D P G N A L P S A V K L A Y S P A S K T F E S Y R V M T Q V H  
34 V E K N I T V T A S V D P P V I D L L Q A D G N A L P S A V K L A Y S P A S K T F E S Y R V M T Q V H

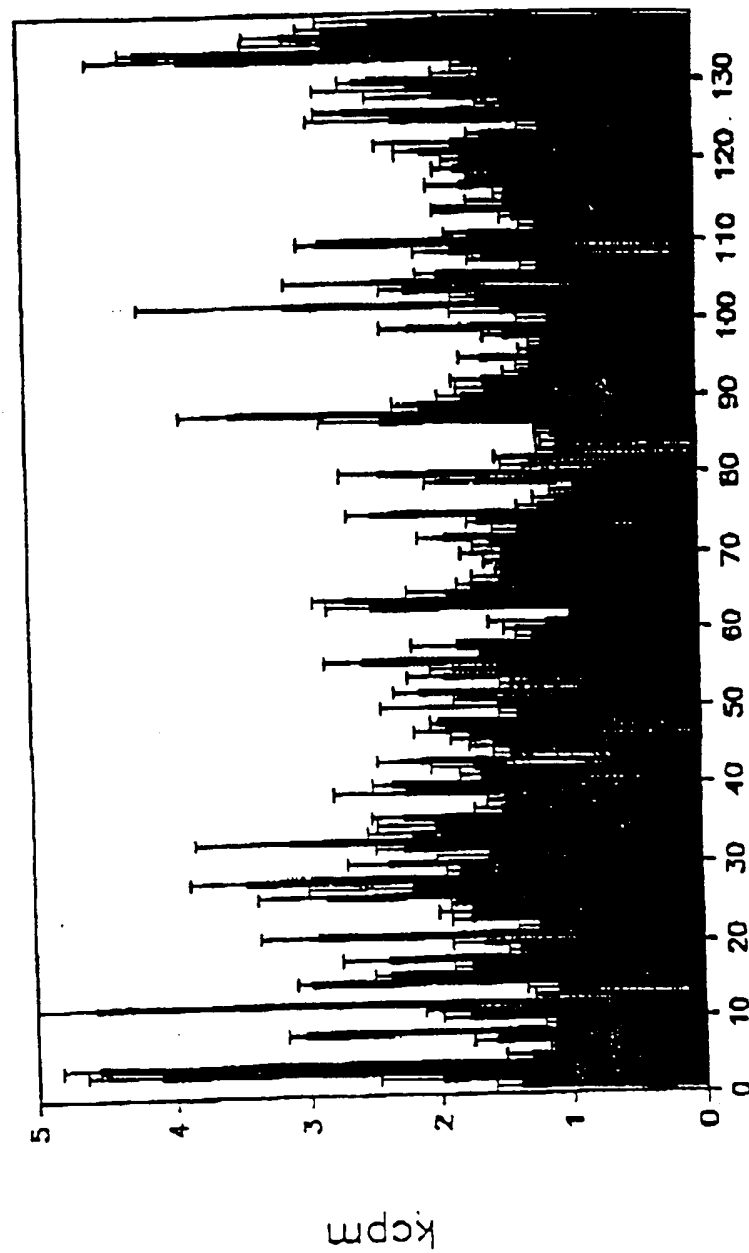
282 T H D A T K K V I V K L A D T P Q L T D V L N S T V Q M P I S V S N G G Q V L S T T A K E F E A A A 100  
184D T H D A T K K V I V K L A D T P Q L T D V L N S T V Q M P I S V S N G G Q V L S T T A K E F E A A A  
34 T H D A T K K V I V K L A D T P Q L T D V L N S T V Q M P I S V S N G G Q V L S T T A K E F E A A A

282 L G Y S A S G V N G V S S S Q E L V I S A A P K T A G T A P T A G N Y S G V V S L V M T L G S 147  
184D L G Y S A S G V N G V S S S Q E L V I S A A P K T A G T A P T A G N Y S G V V S L V M T L G S  
34 L G Y S A S G V N G V S S S Q E L V I S A A P K T A G T A P T A G N Y S G V V S L V M T L G S

Figure 18

monkey 184 D (#11)

# PROLIFERATIVE RESPONSES TO CFA/I SYNTHETIC PEPTIDE



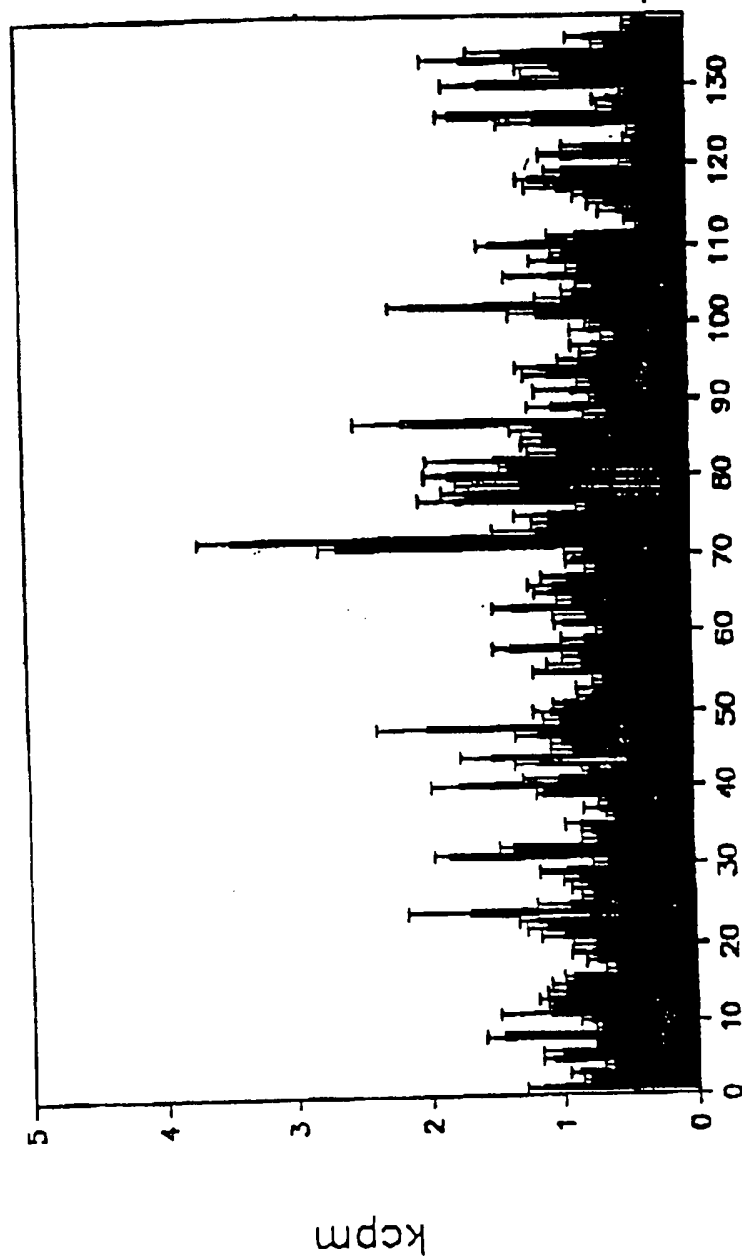
SYNTHETIC CFA/I DECAPEPTIDE, BEGINNING POSITION NUMBER FROM CFA/I SEQUENCE

Figure 19

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*Monkey 34 (#2)*

## PROLIFERATIVE RESPONSES TO CFA/I SYNTHETIC PEPTIDE



SYNTHETIC CFA/I DECAPEPTIDE, BEGINNING POSITION NUMBER FROM CFA/I SEQUENCE

Figure 2a



Monkey 222(#3)

# PROLIFERATIVE RESPONSES TO CFA/I SYNTHETIC PEPTIDE

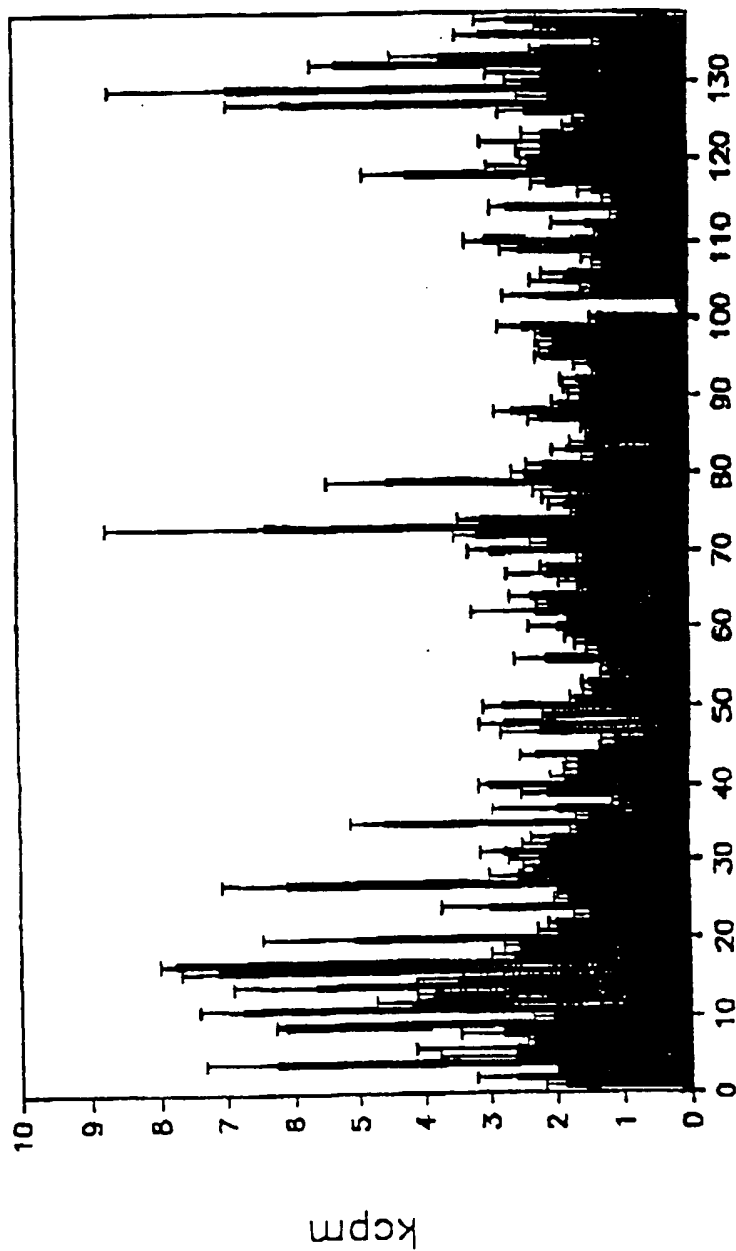


Figure 21

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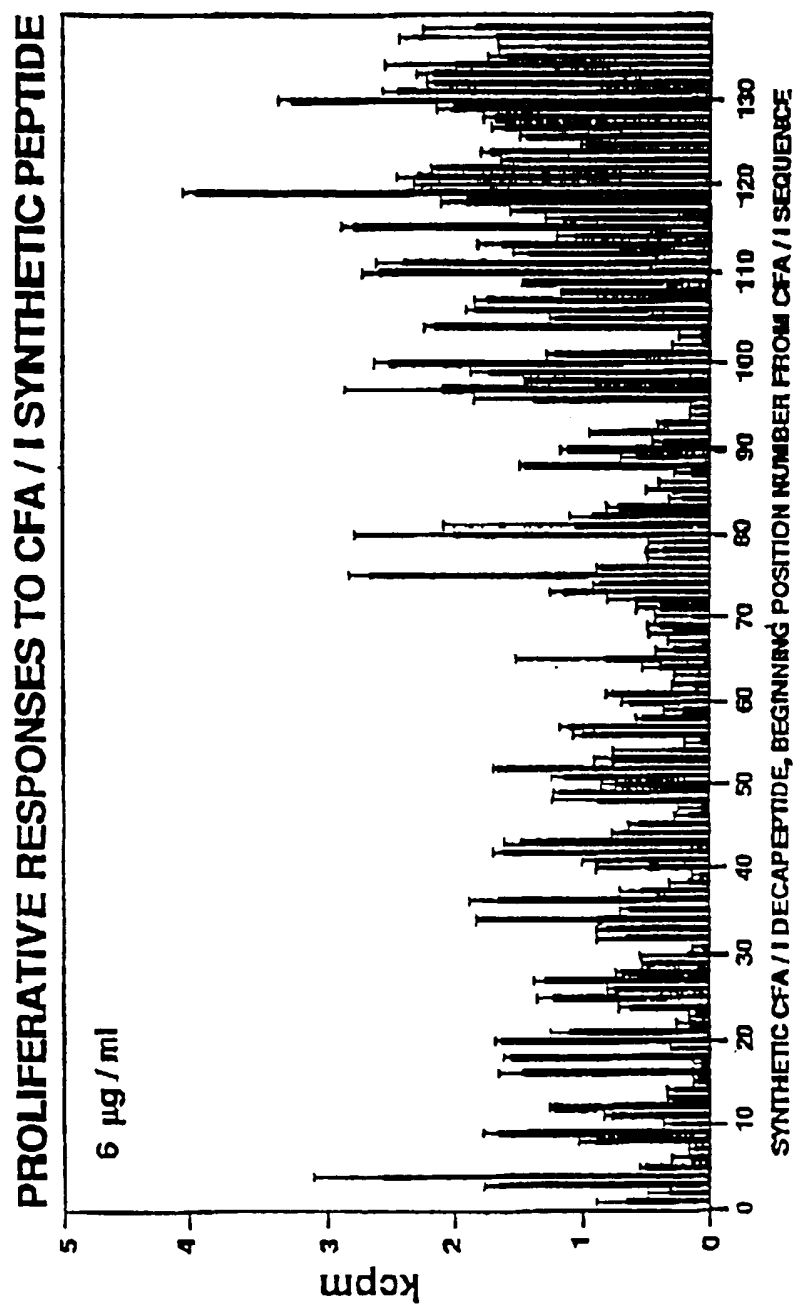


Figure 22

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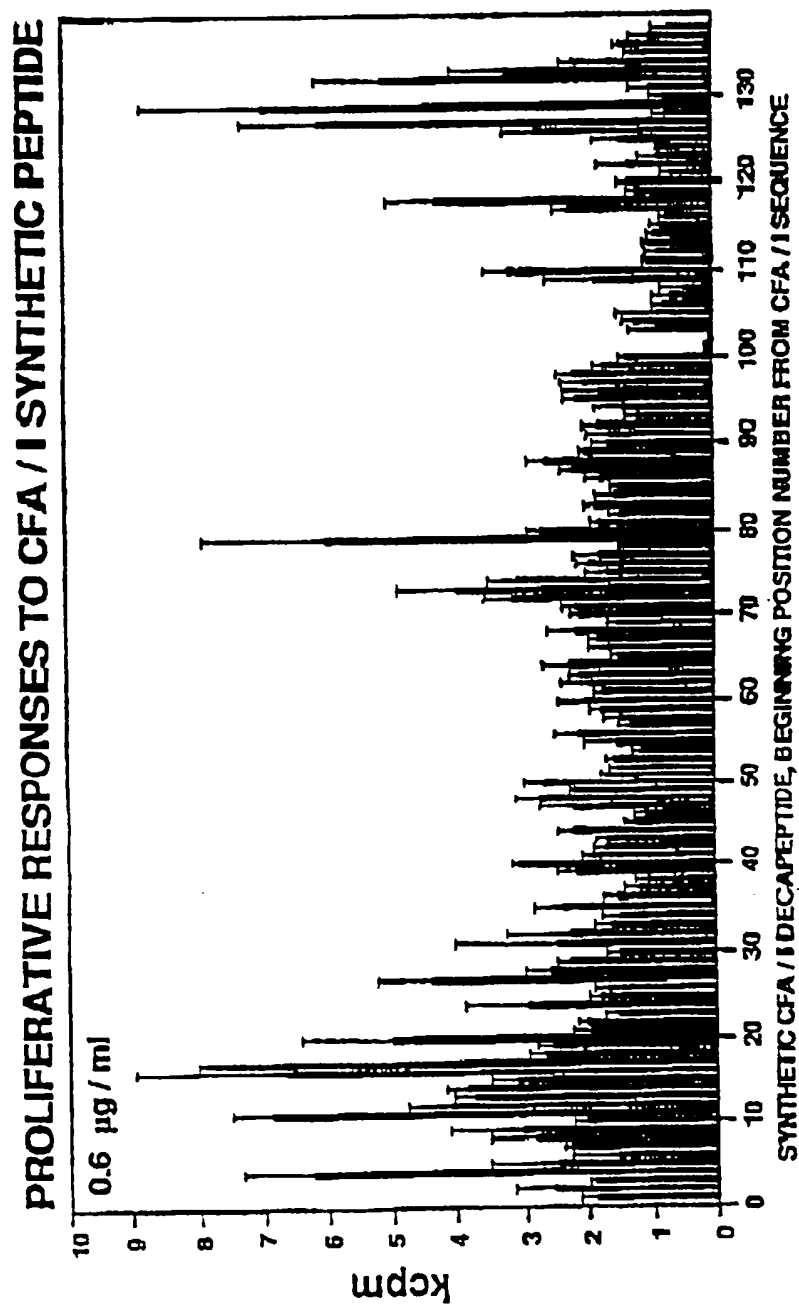


Figure 23

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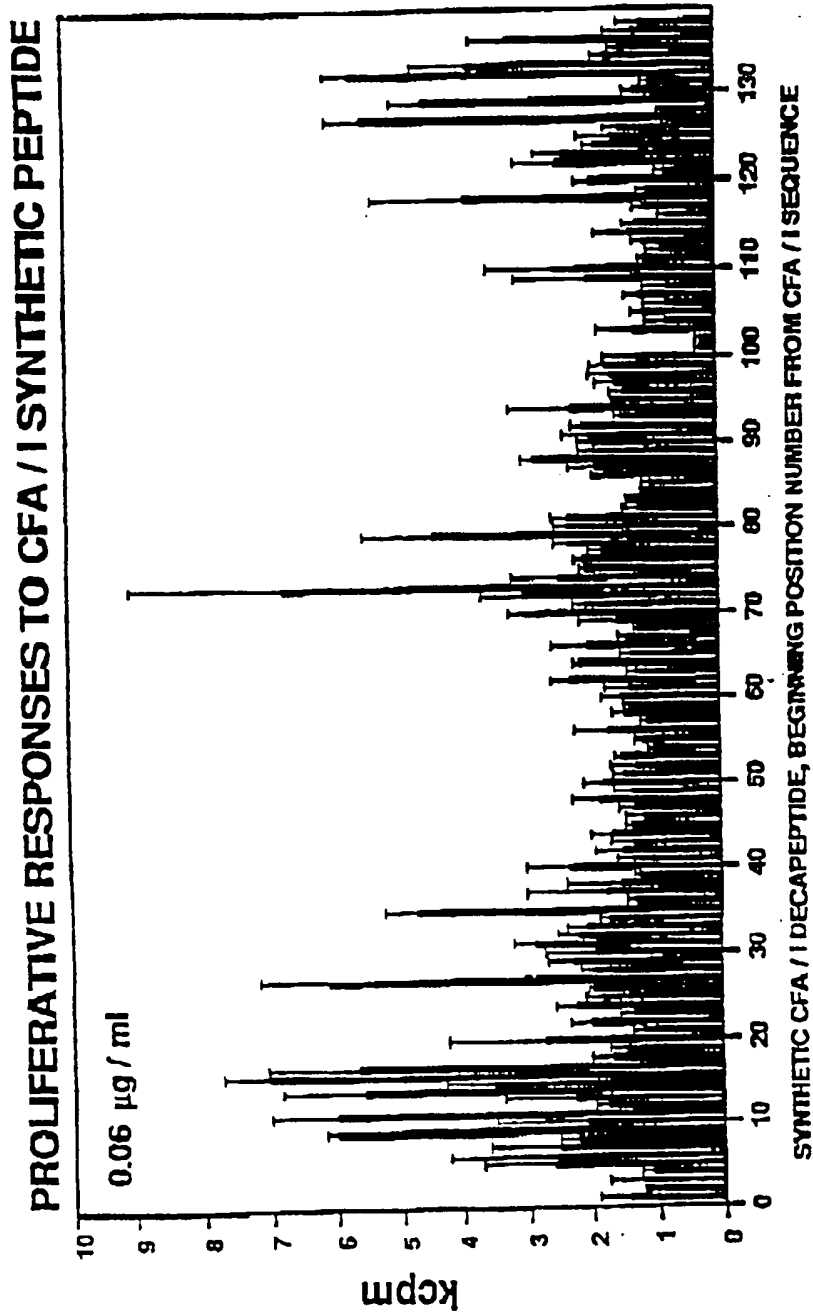


Figure 24

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kcpm

RABBITS AT DAY 31 FOLLOWING ID IMMUNIZATION ON  
DAYS 0, 7, 14, 21 WITH AF/RI IN MICROSPHERES

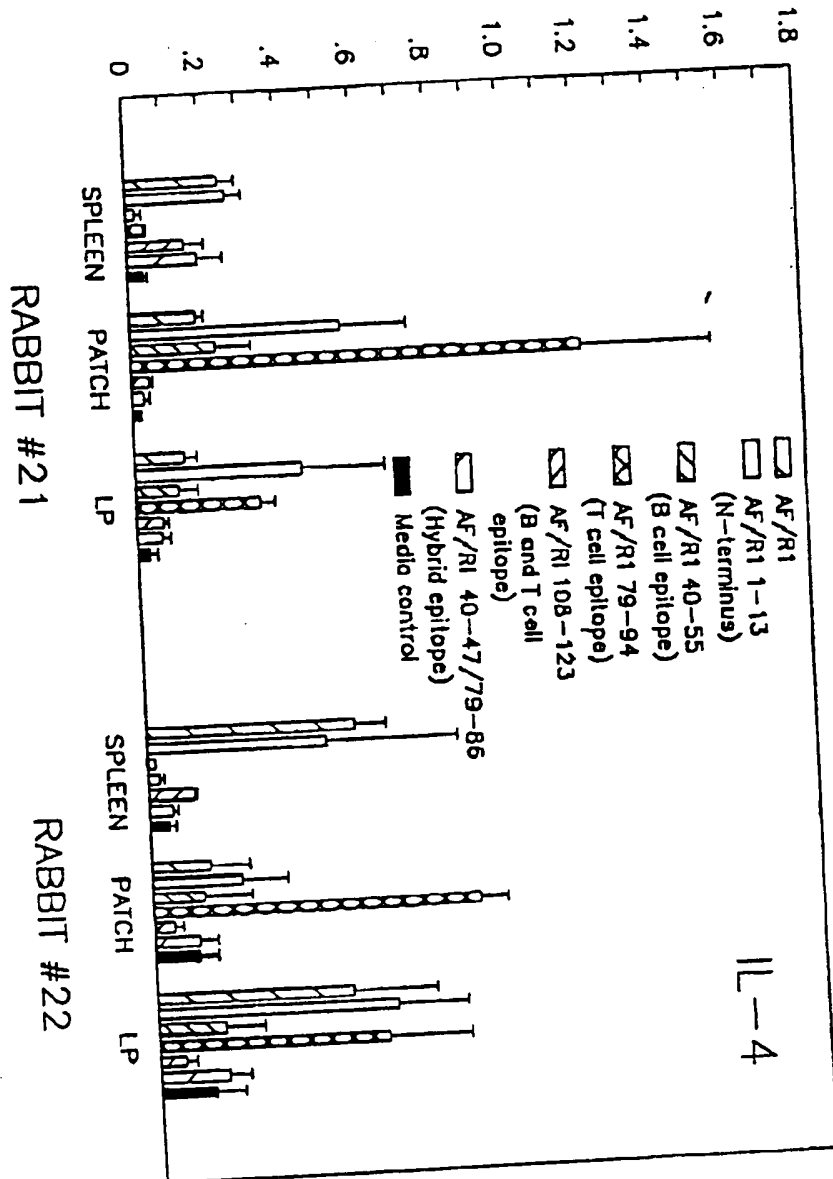


Figure 25

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# RDEC-1 COLONIZATION

## Immunized vs Unimmunized

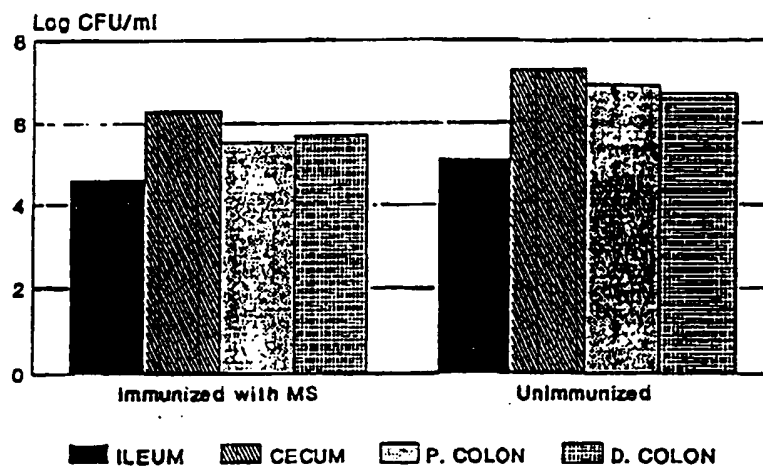
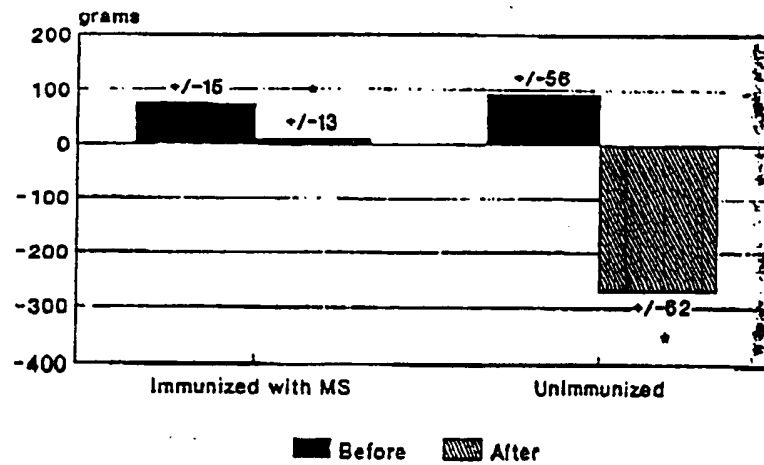


Figure 26

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# WEIGHT CHANGES

Immunized vs Unimmunized

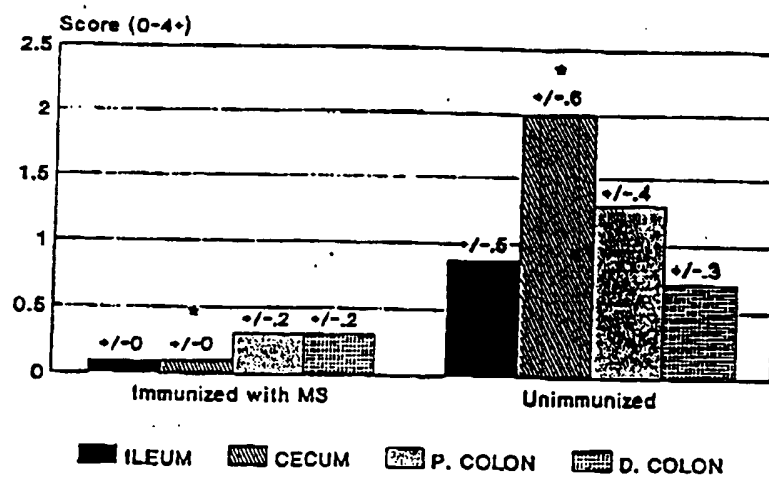


-P&lt;.001

Figure 27

## RDEC-1 ATTACHMENT

Immunized vs Unimmunized



\* P&lt;01

Figure 28



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# Particle Size Distribution

CFA/II Microsphere Vaccine; Lot #L74F2

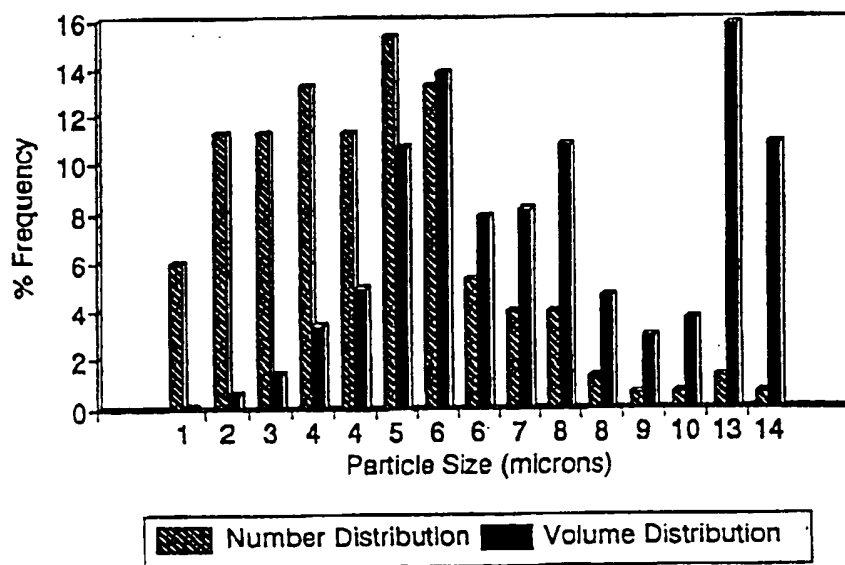


Figure 29

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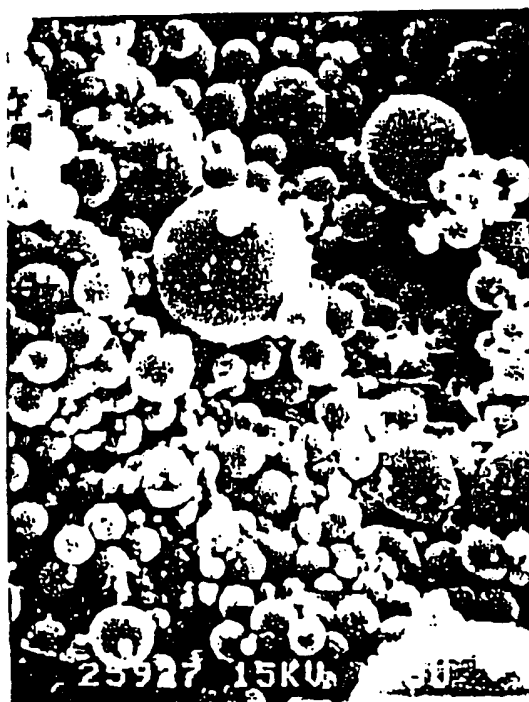


Figure 30

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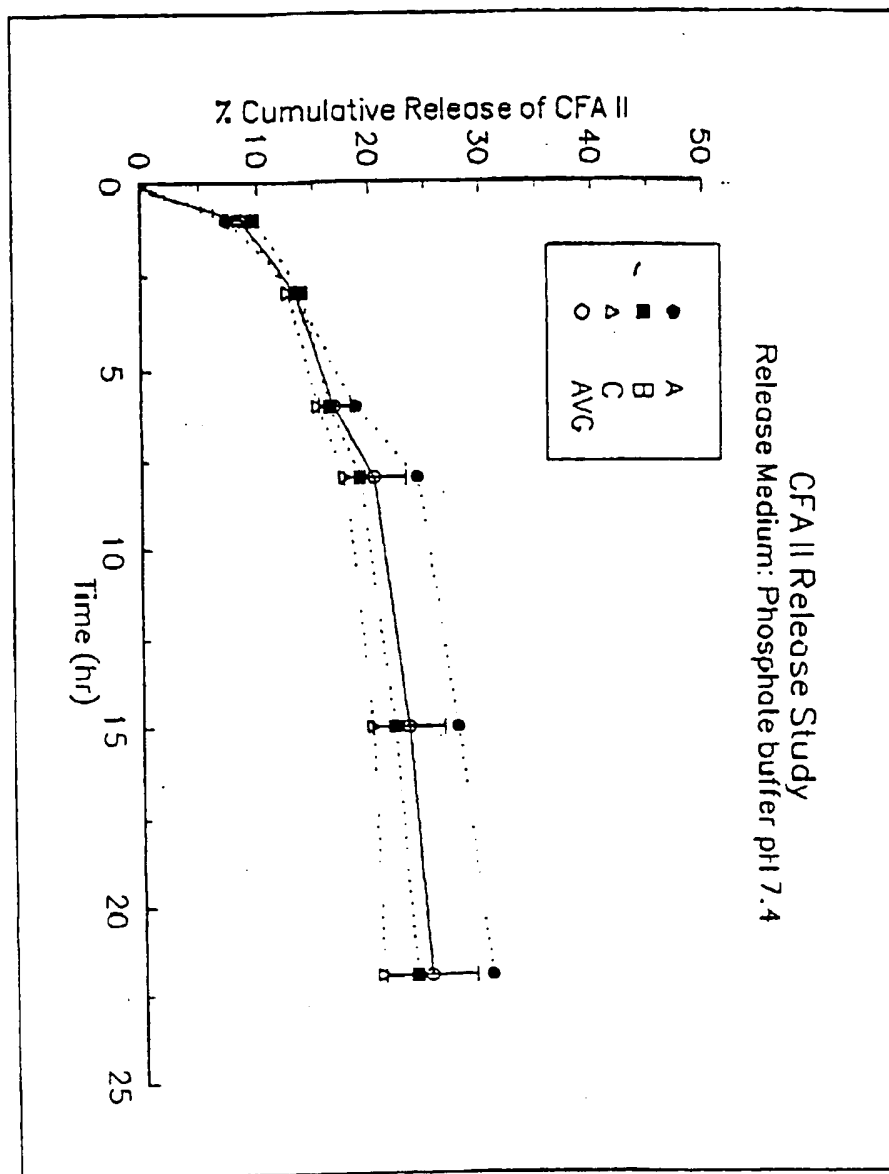


Figure 31

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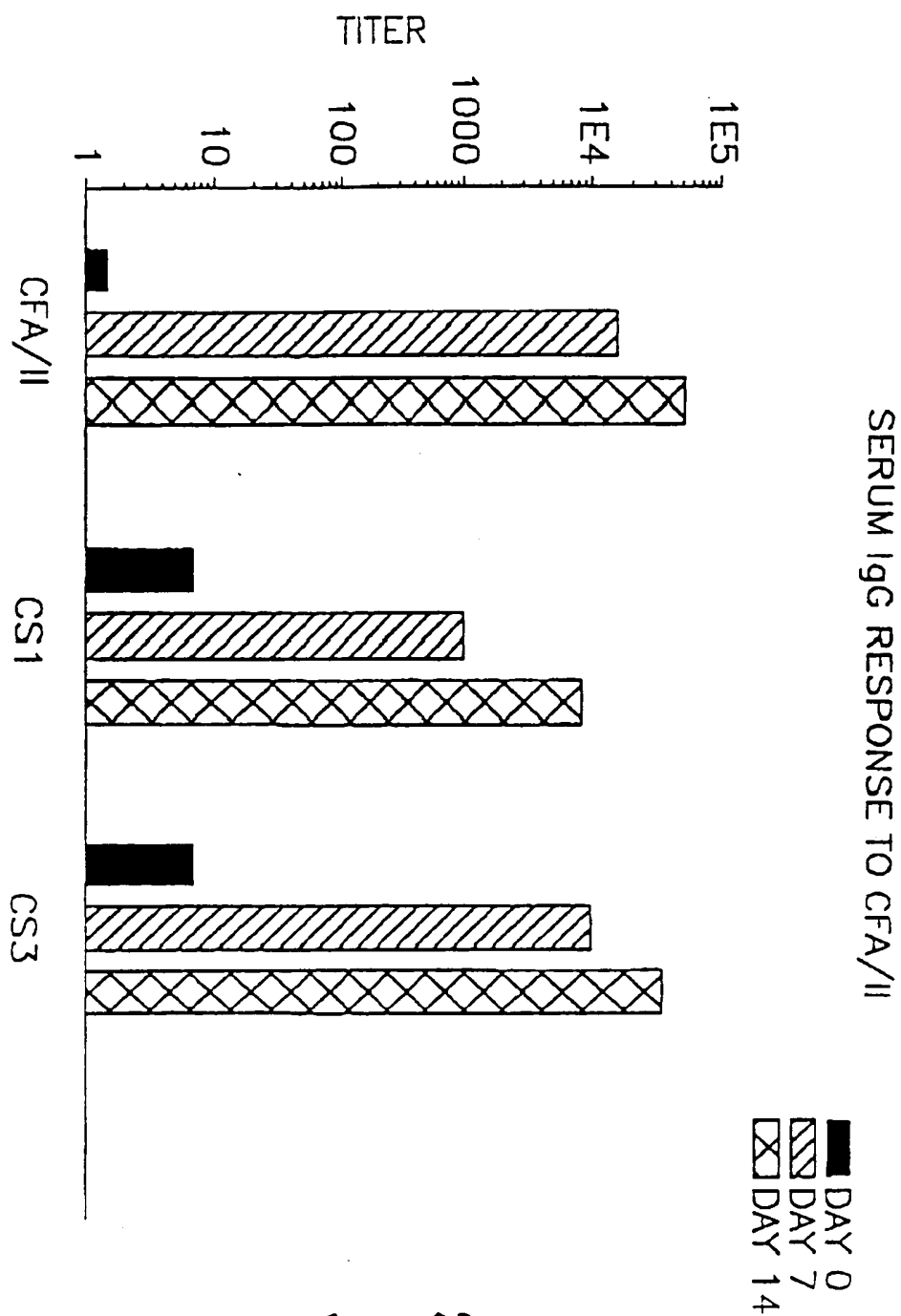
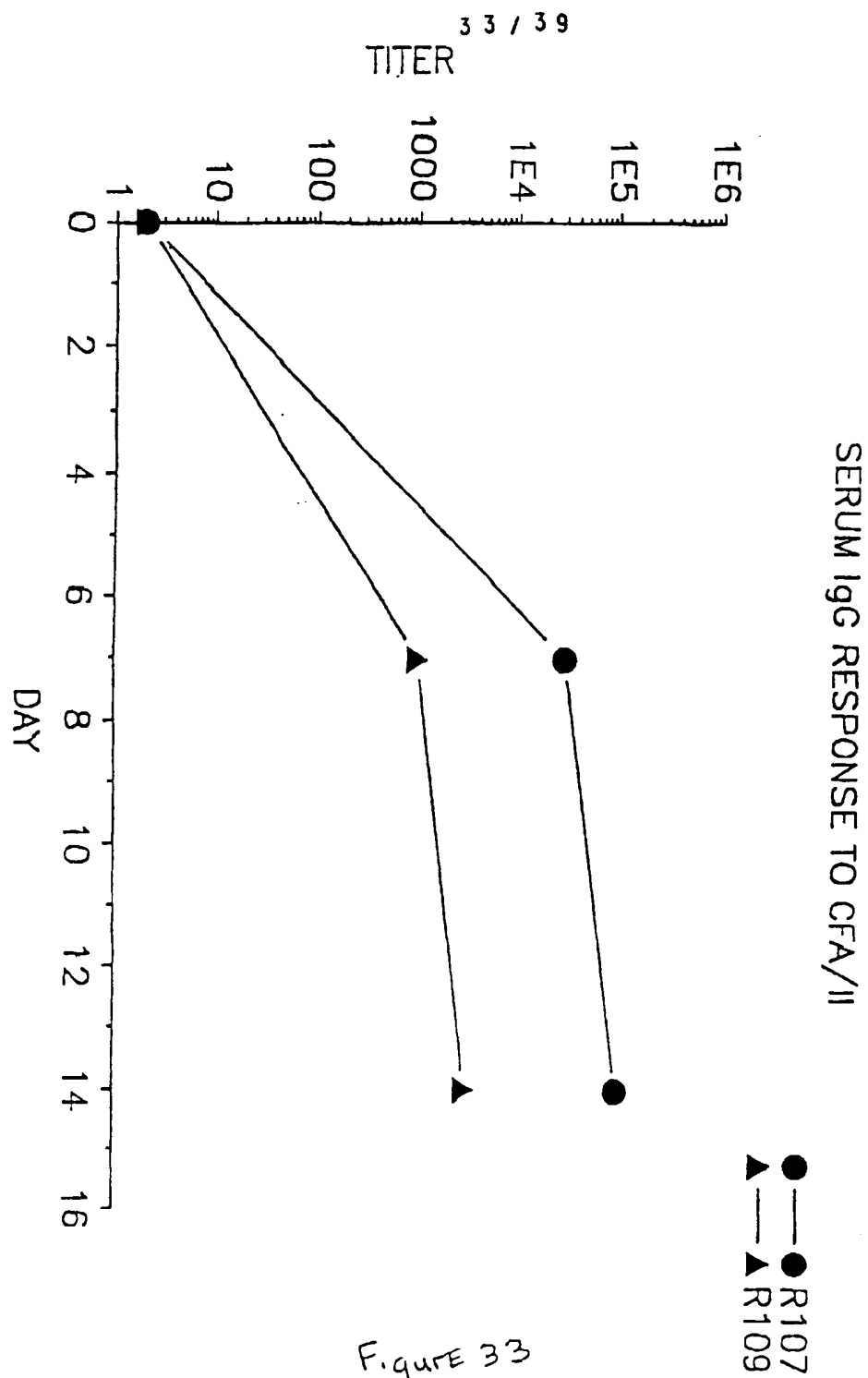
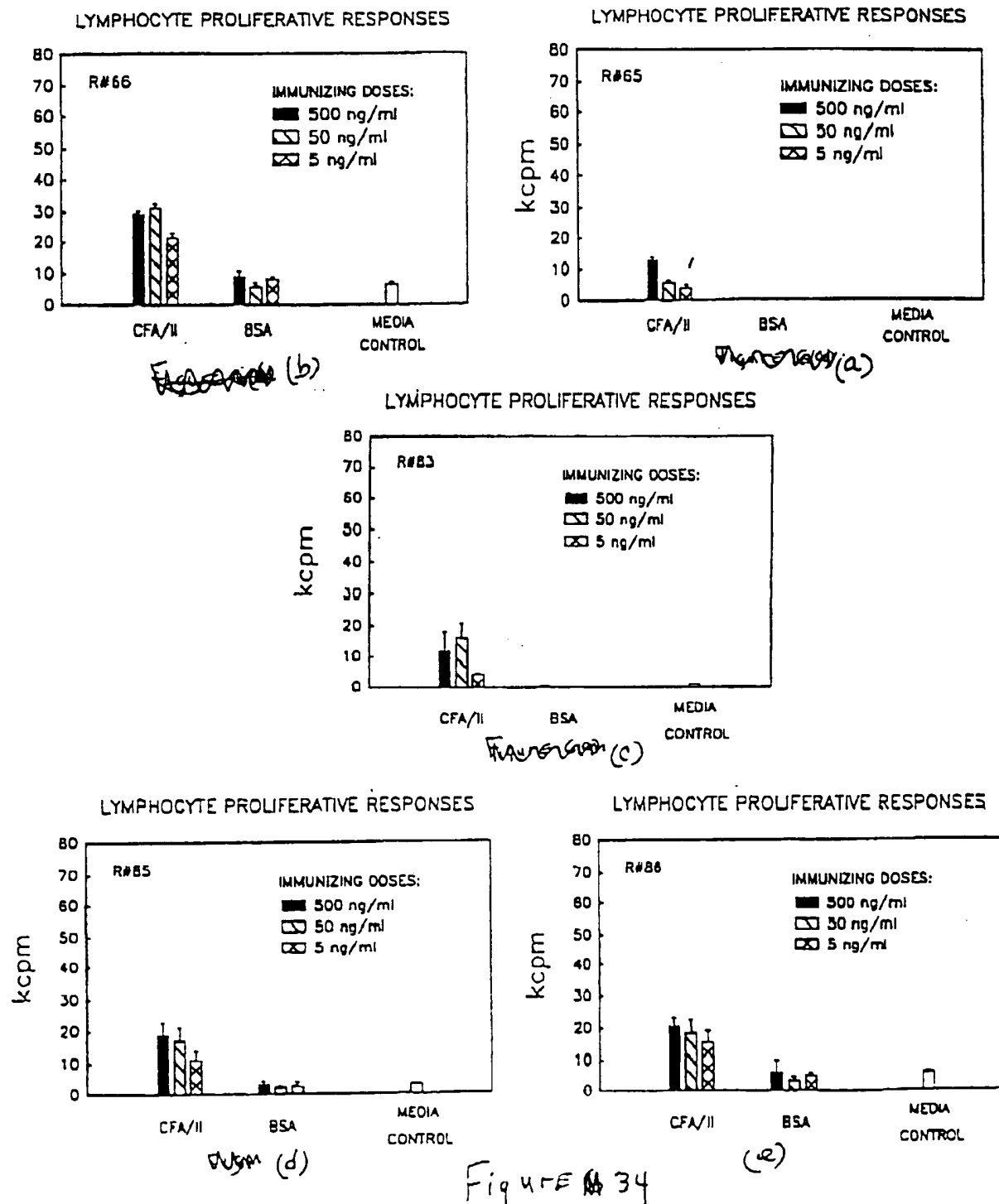


Figure 32

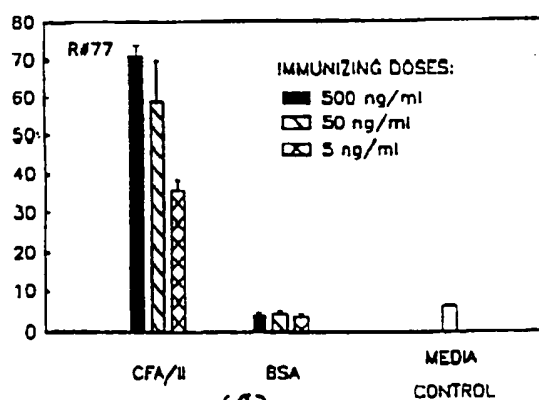


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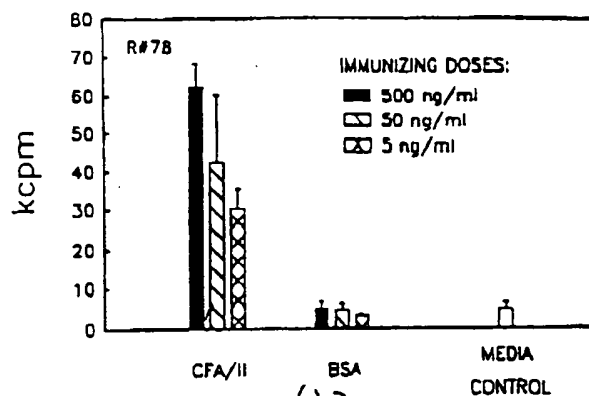
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## LYMPHOCYTE PROLIFERATIVE RESPONSES



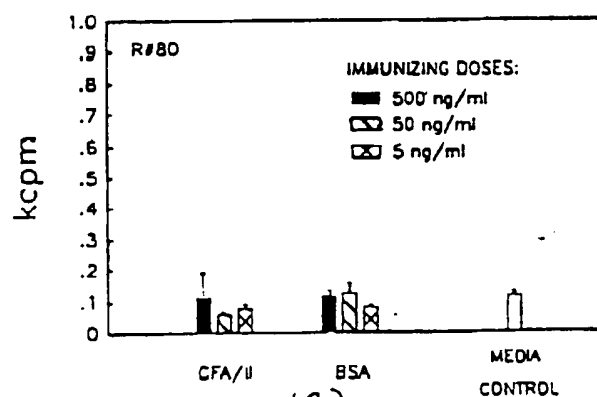
(a)

## LYMPHOCYTE PROLIFERATIVE RESPONSES



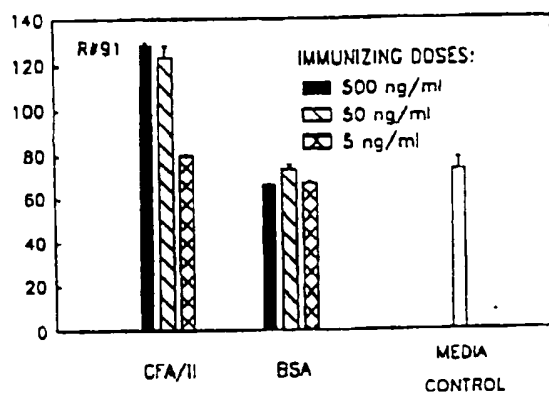
(b)

## LYMPHOCYTE PROLIFERATIVE RESPONSES



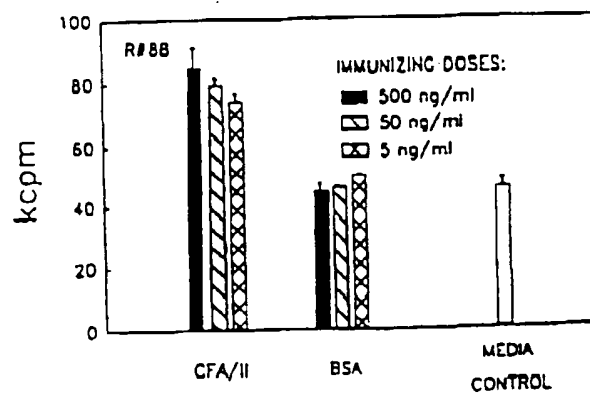
(c)

## LYMPHOCYTE PROLIFERATIVE RESPONSES



(d)

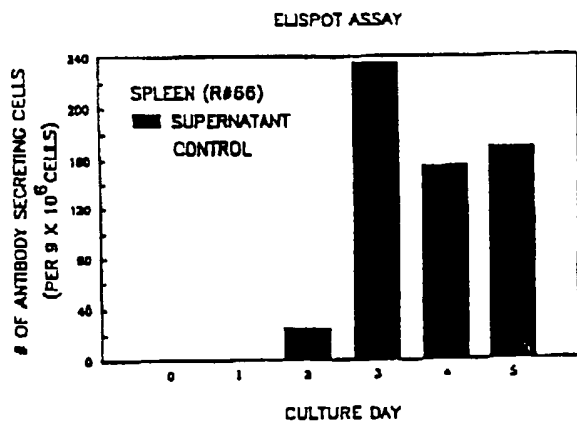
## LYMPHOCYTE PROLIFERATIVE RESPONSES



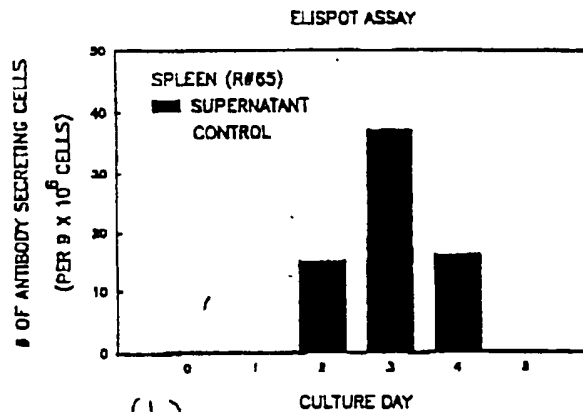
(e)

Figure 35

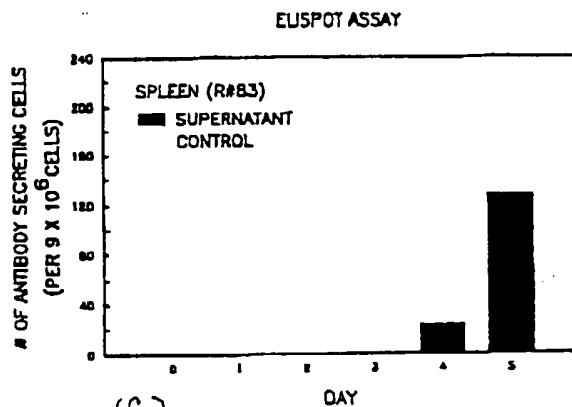
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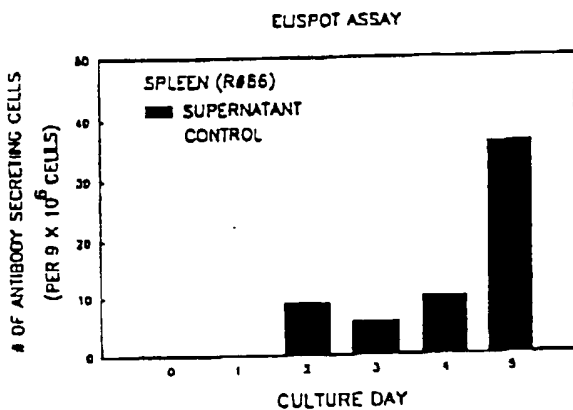
(a)



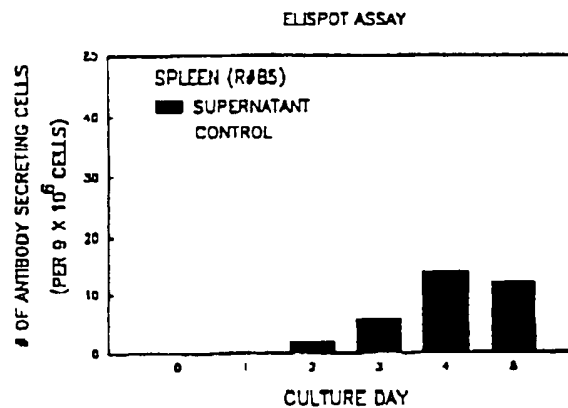
(b)



(c)



(d)



(e)



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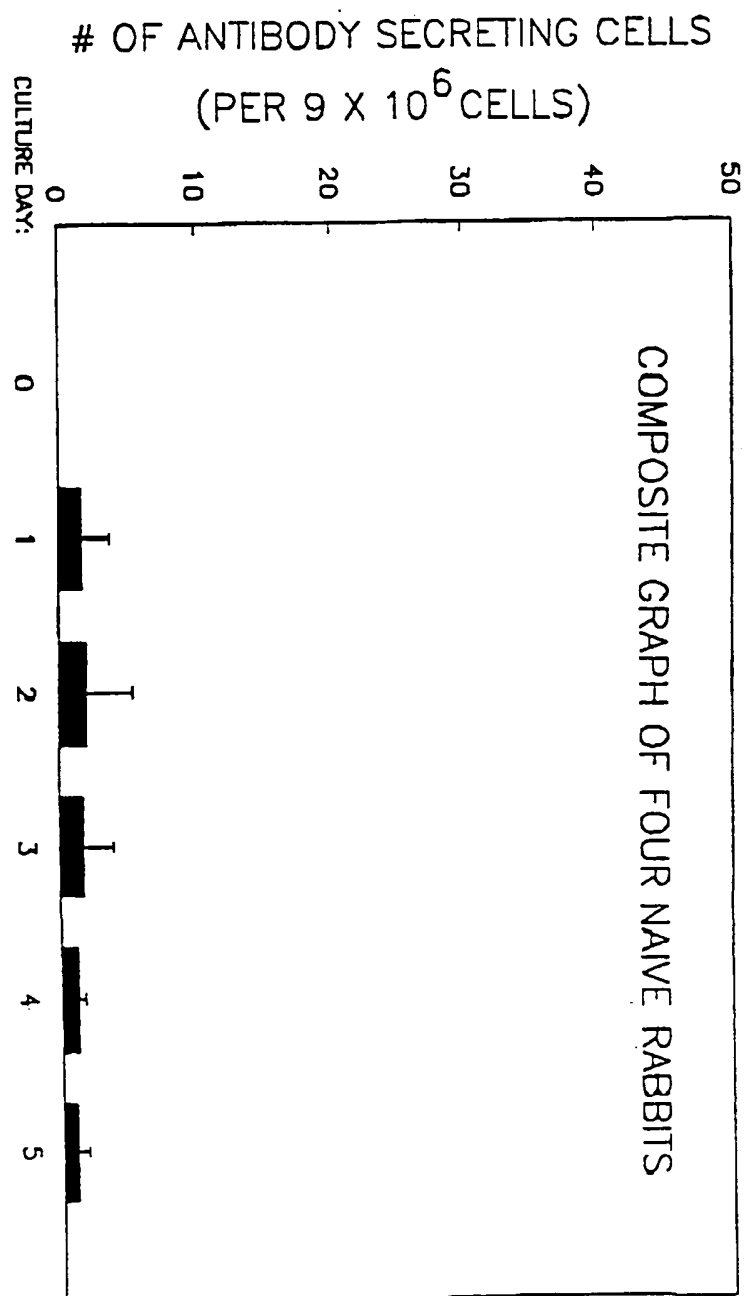


Figure 37

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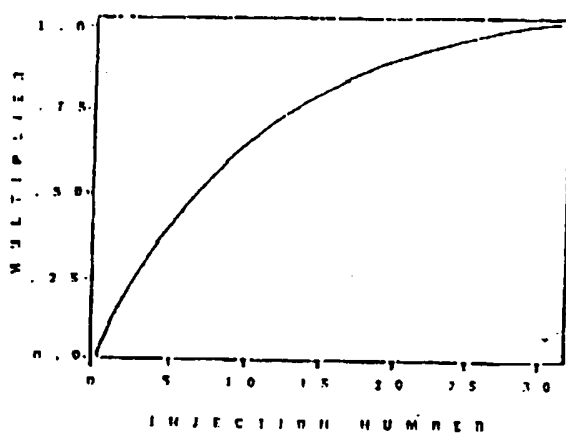


Figure 38

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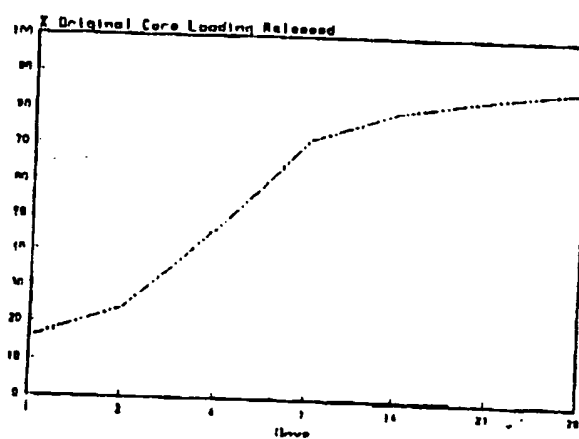


Figure 39

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/02536

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 39/02, 9/26

US CL : 424/85, 88, 89, 92, 417, 422, 450, 458, 469

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85, 88, 89, 92, 417, 422, 450, 458, 469

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

GLUCOID (P) LACTIDE (P) ORAL

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 4,897,268 (TICE ET AL.) 30 JANUARY 1990, COLUMN 2, LINE 6 TO COLUMN 3, LINE 25.	1-21



Further documents are listed in the continuation of Box C.



See patent family annex.

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*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

16 JUNE 1994

Date of mailing of the international search report

JUN 23 1994

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Washington, D.C. 20231

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